ONCOLYTIC ADENOVIRUSES FOR GYNECOLOGIC CANCER

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Academic Dissertation
To be publicly discussed with permission of
the Faculty of Medicine of the University of Helsinki,
in Biomedicum Lecture Hall 3, Haartmaninkatu 8, Helsinki
on March 30th, 2007, at 12 noon

Helsinki 2007

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ISBN 978-952-92-1720-5 (paperback)
ISBN 978-952-10-3787-0 (PDF)
http://ethesis.helsinki.fi

Helsinki 2007
Helsinki University Press

Esse, non videri; voluptatem non rogandum.
[Be, don't pretend; pleasure is not the question.]

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals.

I G. J. Bauerschmitz, D. M. Nettelbeck, A. Kanerva, A. H. Baker, A. Hemminki, P. N. Reynolds and D. T. Curiel. The flt-1 promoter for transcriptional targeting of teratocarcinoma. Cancer Res. 2002 Mar 1;62(5):1271-4

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II G. J. Bauerschmitz, J. T. Lam, A. Kanerva, K. Suzuki, D. M. Nettelbeck, I. Dmitriev, V. Krasnykh, G. V. Mikheeva, M. N. Barnes, R. D. Alvarez, P. Dall, R. Alemany, D. T. Curiel, A. Hemminki. Treatment of ovarian cancer with a tropism modified oncolytic adenovirus. Cancer Res. 2002 Mar 1;62(5):1266-70

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III G. J. Bauerschmitz, A. Kanerva, M. Wang, D. R. Shaw, T. V. Strong, R. A. Desmond, P. Dall, D. T. Curiel and A. Hemminki. Evaluation of a selectively oncolytic adenovirus for local and systemic treatment of cervical cancer. Int J Cancer. 2004 Aug 20; 111(2): 303-9

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IV G. J. Bauerschmitz, K. Guse, A. Kanerva, A. Menzel, I. Herrmann, R. A. Desmond, M. Yamamoto, D. M. Nettelbeck, T. Hakkarainen, P. Dall, D. T. Curiel, A. Hemminki. Triple targeted oncolytic adenoviruses featuring the Cox2 promoter, E1A transcomplementation and serotype chimerism for enhanced selectivity for ovarian cancer cells. Mol Ther. 2006 Aug;14(2):164-74

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ABBREVIATIONS

Ad	adenovirus
Ad3	adenovirus serotype 3
Ad5	adenovirus serotype 5
ADP	adenoviral death protein
ATCC	American Type Culture Collection
bp	basepair
Ca	cancer
CAR	coxsackie-adenovirus receptor
CD	cytosine deaminase
cGMP	current good manufacturing practices
cox-2	cyclooxygenase-2
CR2	constant region 2
CRAd	conditionally replicating adenovirus
C-terminal	carboxy-terminal
delta2	deletion of 3 bp in E1A region
delta24	deletion of 24 bp in CR2 of E1A
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
Flt-1	vascular endothelial growth factor receptor type 1
GM	growth medium
GCV	ganciclovir
6-His	six histidine amino acid residues
HSV-TK	herpes simples virus type I thymidine kinase
hTERT	human telomerase reverse transcriptase
IFN	interferon
Ig	immunoglobulin
i.a.	intra-arterial
i.p.	intraperitoneal
i.t.	intratumoral
i.v.	intravenous
kb	kilobase
kD	kiloDalton
luc	firefly luciferase
MHC I	major histocompatibility complex I
mock	control without virus
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2Htetrazolium
pfu	plaque forming unit
Rb	retinoblastoma
RGD(-4C)	arginine-glycine-aspartic acid (with four cysteins)
s.c.	subcutaneous
TK	thymidine kinase
TSP	tumor/tissue specific promoter
vp	viral particle

ABSTRACT

Gene therapy is a promising novel approach for treating cancers resistant to or escaping currently available modalities. Treatment approaches are based on taking advantage of molecular differences between normal and tumor cells. Various strategies are currently in clinical development with adenoviruses as the most popular vehicle. Recent developments include improving targeting strategies for gene delivery to tumor cells with tumor specific promoters or infectivity enhancement. A rapidly developing field is as well replication competent agents, which allow improved tumor penetration and local amplification of the anti-tumor effect. Adenoviral cancer gene therapy approaches lack cross-resistance with other treatment options and therefore synergistic effects are possible.

This study focused on development of adenoviral vectors suitable for treatment of various gynecologic cancer types, describing the development of the field from non-replicating adenoviral vectors to multiple-modified conditional replicating viruses.

Transcriptional targeting of gynecologic cancer cells by the use of the promoter of vascular endothelial growth factor receptor type 1 (flt-1) was evaluated. Flt-1 is not expressed in the liver and thus an ideal promoter for transcriptional targeting of adenoviruses. Our studies implied that the flt-1 promoter is active in teratocarcinomas and therefore a good candidate for development of oncolytic adenoviruses for treatment of this often problematic disease with then poor outcome.

A tropism modified conditionally replicating adenovirus (CRAd), Ad5-Δ24RGD, was studied in gynecologic cancers. Ad5-Δ24RGD is an adenovirus selectively replication competent in cells defective in the p16/Rb pathway, including many or most tumor cells. The fiber of Ad5-Δ24RGD contains an integrin binding arginine-glycine-aspartic acid motif (RGD-4C), allowing coxackie-adenovirus receptor independent infection of cancer cells. This approach is attractive because expression levels of CAR are highly variable and often low on primary gynecological cancer cells. Oncolysis could be shown for a wide variety of ovarian and cervical cancer cell lines as well as primary ovarian cancer cell spheroids, a novel system developed for *in vitro* analysis of CRAds on primary tumor substrates. Biodistribution was evaluated and preclinical safety data was obtained by demonstrating lack of replication in human peripheral blood mononuclear cells. The efficicacy of Ad5-Δ24RGD was shown in different orthotopic murine models including a highly aggressive intraperitoneal model of disseminated ovarian cancer cells, where Ad5-Δ24RGD resulted in complete eradication of intraperitoneal disease in half of the mice.

To further improve the selectivity and specificity of CRAds, triple-targeted oncolytic adenoviruses were cloned, featuring the cyclo-oxygenase-2 (cox-2) promoter, E1A transcomplementation and serotype chimerism. Those viruses were evaluated on ovarian cancer cells for specificity and oncolytic potency with regard to two different cox2 versions and three different variants of E1A (wild type, delta24 and delta2delta24). Ad5/3cox2Ld24 emerged as the best combination due to enhanced selectivity without potency lost *in vitro* or in an aggressive intraperitoneal orthotopic ovarian tumor model.

In summary, the preclinical therapeutic efficacy of the CRAds tested in this study, taken together with promising biodistribution and safety data, suggest that these CRAds are interesting candidates for translation into clinical trials for gynecologic cancer.

REVIEW OF THE LITERATURE

1. Introduction

After cardiovascular disease, cancer is the second most common cause of death in Europe, and is currently responsible for ca. a fourth of all deaths. Whereas in younger women cervical cancer is among the most frequent ones, with increasing age the incidences of cancer of the uterine corpus or the ovaries increase and the latter becomes an important cause of mortality despite numerous innovations in therapy concepts (2003; Jemal, Siegel et al. 2006).

For operable patients, surgery is the cornerstone of gynecological cancer treatment; confirmation of histology, adequate staging and maximal cytoreduction being the goals. Thus, surgical debulking or complete removal of the tumor is a requisite for a chance of curative treatment for nearly all types of gynecologic cancer. Some cases of cervical or endometrial cancer can be effectively treated with radiation or chemoradiation alone. Here, progress in anaesthesia, allowing surgery in patients with reduced performance levels, combined with improved surgical techniques, have made surgery available to more patients (Marnitz, Kohler et al. 2006).

Innovations in the field of radiation with new or improved radiation sources, and three dimensional planning as well as advanced chemotherapy regimes with new therapeutics have improved the outcome of gynecologic cancer (Ozols 2006; Ozols 2006). More effective combination regimens (Ozols 2006; Ozols 2006) and different application routes such as intraperitoneal chemotherapy have been suggested to improve the outcome of advanced ovarian cancer (Armstrong, Bundy et al. 2006; Markman and Walker 2006; Ozols, Bookman et al. 2006).

Taken together, outcomes in terms of disease-free and overall survival have improved for early stages, but women with metastatic disease remain often incurable (Jemal, Siegel et al. 2006). Targeted therapies might help to overcome this obstacle, although due to an absence of a common driving oncogene, single targeted therapies are unlikely to yield significant benefit (Darcy and Schilder 2006).

In recent decades, an intense basic research effort has begun to reveal the nature of cancer as a disease of genes. Specifically, epigenetic and genetic alterations of tumor suppressor and oncogenes are the cause of cancer. These changes can be hereditary, or – more commonly – arise during the lifetime of an individual. A logical result of these findings is the idea of correcting the molecular defects. Alternatively, these differences could be used for targeting an antitumor effect to malignant cells. Thus, molecularly targeted therapies stem from our ability to detect molecular defects that set cancer cells apart from normal tissues (Hemminki 2002; Chon, Hu et al. 2006; Tuve, Wang et al. 2006). Cancer gene therapy includes a wide variety of heterogeneous approaches for which the common denominator is transfer of genes, which then code for the proteins or in some cases complete viruses that deliver the anti-tumor effect (Hauses and Schackert 2000; Vorburger and Hunt 2002; Cross and Burmester 2006).

Gene transfer is performed by different vector systems with respective advantages and disadvantages. The specifics of these vector systems make them suitable for the treatment of either monogenic disorders, aquired diseases or for cancer. Whereas the treatment of the former two typically benefits from long-term gene expression and thus a stable non-immongenic gene transfer method, gene therapy for cancer requires effective high level transduction and gene expression in target cells. Currently, the most common vector systems

are adenoviruses (Ad), retroviruses including lentiviruses, adeno-associated viruses (AAV) and non-viral gene transfer systems.

2. Vehicles for gene transfer

A traditional vehicle is the retrovirus, which integrates into the host cell genome and can therefore achieve lasting gene expression. However, its major disadvantages are the possibility of insertion mutagenesis/oncogenesis, low transduction efficacy, difficulties in production of high titers and infection of only cycling cells (Luther-Wyrsch, Costello et al. 2001; Pandya, Klimatcheva et al. 2001; Ross, Wright et al. 2001). For these reasons, retroviruses may be most suitable for treatment of diseases where long term gene expression is required. Lentiviruses may overcome some of these problems but their clinical safety or effifacy have not yet been demonstrated. Adeno-associated viruses are non-pathogenic single-stranded DNA viruses which, when wild type, may integrate into the human chromosome 19 and cause long term gene expression, whereas recombinants stay mostly episomal (Ponnazhagan, Curiel et al. 2001). Early preclinical studies show encouraging results (Vermeij, Zeinoun et al. 2001). However, recent findings suggest that adeno-associated viruses may cause deletions or changes in chromosome 19 (Miller, Rutledge et al. 2002). Finally, non-viral vectors are undergoing evaluation. Liposome vector systems are cationic complexes where transgenes are carried inside a lipid double-membrane, which can be modified to bind selectively to a specific target receptor. The liposomes enter their target cell via endocytosis and release subsequently the DNA-load into the cell. For treatment of cancer, a major problem has been low transduction efficiency, particularly prominent *in vivo* and further underlined by data obtained from clinical trials (Lee, Yoon et al. 2000; Hortobagyi, Ueno et al. 2001; Rochlitz 2001; Wang, Zhang et al. 2001; Yoo, Hung et al. 2001; Chon, Hu et al. 2006; Cross and Burmester 2006).

3. Cancer gene therapy approaches

Gene therapy for cancer can be divided into at least six different categories: 1) mutation compensation, 2) molecular chemotherapy, 3) genetic immunopotentation, 4) genetic modulation of resistance/sensitivity, 5) oncolytic (conditionally replicating) viruses and 6) antiangiogenic gene therapy. The goal of mutation compensation is correction of a crucial molecular change within cancer cells. For example, mutations of tumor suppressor genes such as p53 or BRCA1 or overexpression of oncogenes such as erbB-2 are major targets for replacement or inactivation, respectively (Nieto, Cagnoni et al. 2000; Casado, Nettelbeck et al. 2001) (Fig. 1). Molecular chemotherapy, also known as suicide or prodrug conversion gene therapy, is the selective delivery or expression of genes encoding a prodrug-activating enzyme for tumor cell eradication (Fig. 2). Approaches tested in the clinic include vector mediated delivery of herpes simplex virus thymidine kinase (HSV-TK) and *Echerichia coli* cytosine deaminase (*E.coli* CD), which locally convert non-toxic prodrugs (e.g. ganciclovir for HSV-TK or 5-fluorocytosine for *E.coli* CD) into potent cell poisons. Lateral diffusion of the activated drug into untransduced neighboring cells causes additional cell killing and is described as the “bystander effect”. This helps alleviate the daunting task of transduction of each tumor cell (Elshami, Saavedra et al. 1996).

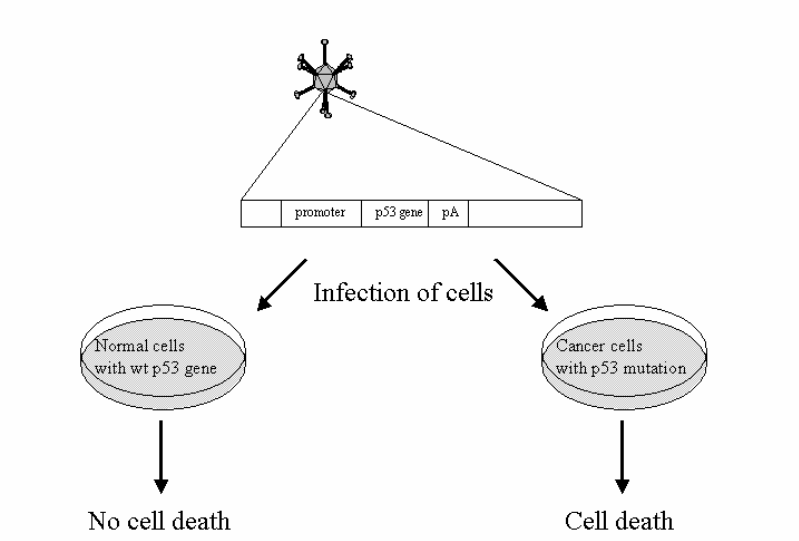

Fig. 1. Mutation compensation: expression of a wild type p53 gene in cells with mutated version of this gene causes cell death selectively in cells with former mutated gene.

In contrast, genetic immunopotentation efforts involve the modification of either immune or tumor cells to augment immunological recognition of neoplastic cells (Nishida, Maeda et al. 2002) (Fig. 3). In a phase III randomized trial in patients with renal cell carcinoma an autologous renal tumor cell vaccine increased the 70 month progression-free survival rates from 59% to 72%. The vaccination was well tolerated and only 12 adverse events out of 379 patients were reported (Jocham, Richter et al. 2004).
In another approach, investigators have utilized a variety of strategies to modify resistance or sensitivity of cells for chemotherapy or radiation in order to enhance the therapeutic index (Duverger, Sartorius et al. 2002; Schiedlmeier, Schilz et al. 2002).
Oncolytic viruses, such as conditionally replicating adenoviruses (CRAds), take advantage of tumor specific changes, which allow preferential replication in and subsequent death of tumor cells.
Finally, antiangiogenic gene therapy targets the development of new vessels in tumor tissue thus inhibiting tumor growth.

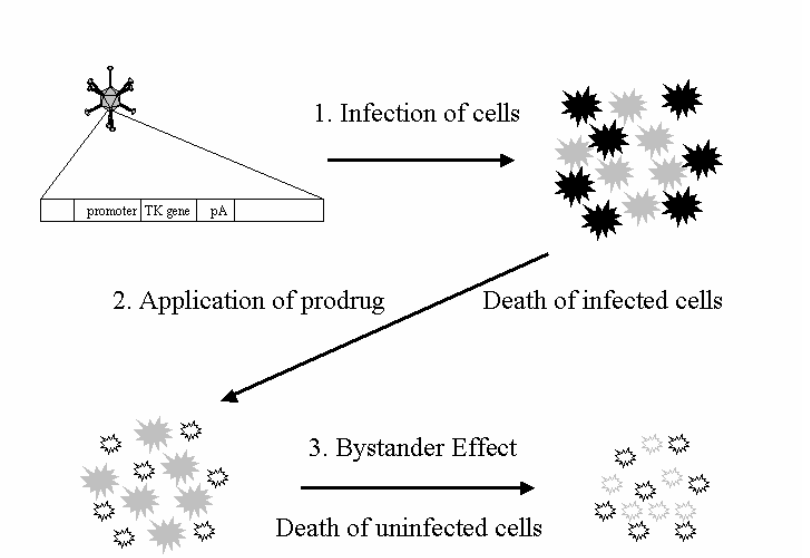

Fig. 2. Suicide gene therapy: After gene delivery with a vector coding for *HSVtk*, administration of ganciclovir causes death of infected cells followed by destruction of surrounding cells due to the bystander effect.

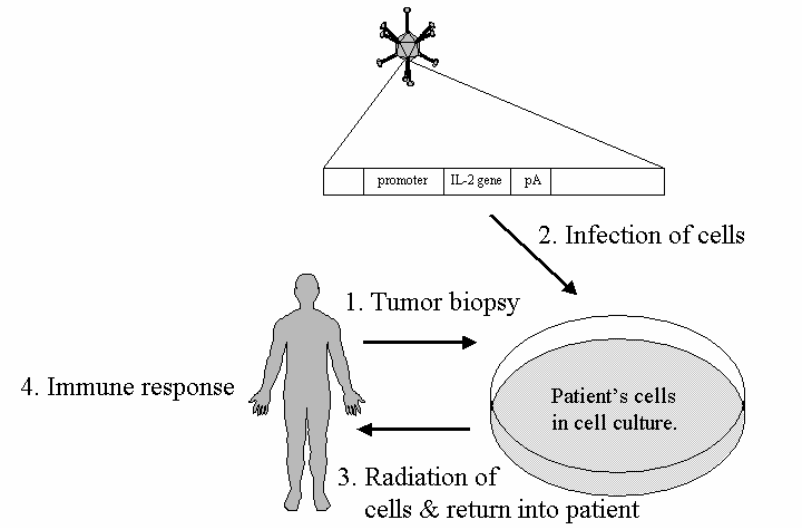

Fig. 3. Immunopotentation: the patient's own cells are extracted and infected in vitro with an interleukin encoding vector, followed by retransfusion of the transfected cells back into patient, causing an immune response to the tumor.

4.1 Adenovirus for gene therapy

Adenoviruses are double-stranded DNA viruses whose major capsid components are hexon, penton and fiber. Adenoviral infection is mediated by binding of the knob region, located at

the carboxy terminus of the fiber, to its corresponding receptor, which is the Coxsackie-Adenovirus Receptor (CAR) for most serotypes. Binding is followed by interaction between cellular integrins and an arginine-glycine-aspartic acid motif (RGD-motif) located at the penton base. This binding leads to formation of endosomes and viral internalization. Subsequently, after this receptor-mediated endocytosis, the virus escapes from the endosome and the adenoviral DNA is transported in the cytoplasma microtubule- and dynein/dynactin-dependent to the nucleus. Then the virus docks to the nuclear core complex receptor, imports in the nucleus and adenoviral protein synthesis, or in case of nonreplicating Ads, transgene expression begins (Meier and Greber 2003). Adenoviral DNA is not regularly integrated into the host genome, thereby resulting in a low risk of mutagenesis. Nevertheless, the limited duration of gene expression may render Ads less desirable for the therapy of diseases where long-term expression is needed, but is adequate for cancer gene therapy approaches, where the purpose typically is to kill the target cells. Infection is not dependent on cell cycle phase; therefore, both cycling and non-dividing cells are infected.

Importantly, a most appealing feature of Ad for cancer therapy is its unparalleled capacity for gene transfer and expression in vivo. Further, production of high titers of cGMP Ad, necessary for clinical trials, is well established. Adenoviral infection of tissues is determined chiefly by the degree of CAR expression (Zabner, Freimuth et al. 1997; Miller, Buchsbaum et al. 1998; Kaner, Worgall et al. 1999; Li, Pong et al. 1999; Walters, Grunst et al. 1999; Fechner, Wang et al. 2000; Okegawa, Li et al. 2000; Wan, Leon et al. 2000; Cohen, Shieh et al. 2001; Cripe, Dunphy et al. 2001; Nalbantoglu, Larochelle et al. 2001; Okegawa, Pong et al. 2001; Seidman, Hogan et al. 2001; Shayakhmetov, Li et al. 2002). The natural tropism of intravascular Ad results in accumulation mainly in the liver, spleen, heart, lung and kidneys (Huard, Lochmuller et al. 1995; van der Eb, Cramer et al. 1998; Reynolds, Dmitriev et al. 1999; Wood, Perrotte et al. 1999; Bilbao, Gerolami et al. 2000). Tissue macrophages, such as Kuppfer cells of the liver, have a major role in clearing Ad from blood REFs. Although CAR is expressed ubiquitously on most normal epithelial tissues, lack or down-regulation of CAR has been reported for various tumor types and may be associated with tumor aggressiveness and could be an ubiquitous phenomenon (Dmitriev, Krasnykh et al. 1998; Hemmi, Geertsen et al. 1998; Miller, Buchsbaum et al. 1998; Kasono, Blackwell et al. 1999; Khuu, Conner et al. 1999; Li, Pong et al. 1999; Vanderkwaak, Wang et al. 1999; Dodson, DeMarzo et al. 2000; Fechner, Wang et al. 2000; Heinicke, Hemmi et al. 2000; Kelly, Miller et al. 2000; Okegawa, Li et al. 2000; Anders, Ding et al. 2001; Cripe, Dunphy et al. 2001; Douglas, Kim et al. 2001; Okegawa, Pong et al. 2001; Seidman, Hogan et al. 2001; Hemminki and Alvarez 2002; Shayakhmetov, Li et al. 2002). Also, recent preliminary findings suggest a connection between CAR function and cell adhesion, perhaps associated with a tumor suppressing effect (Okegawa, Li et al. 2000; Okegawa, Pong et al. 2001), as CAR may be a transmembrane component of tight junctions (Cohen, Shieh et al. 2001). Furthermore, it has been suggested that CAR expression correlates inversely with the tumor stage (Okegawa, Li et al. 2000; Anders M. 2001), and that an over-activity of the *RAS-MAPK* pathway, found in many or most tumors, may cause downregulation of CAR (Anders M. 2001).
Due to the broad tropism of adenoviruses, targeting to tumors could be useful. Two principal means for achieving this goal exist: a) transcriptional targeting and b) transductional targeting. Transcriptional targeting involves genetically limiting the expression of the introduced gene to specific tissues through the use of the promoter sequences of genes upregulated in these tissues (Miller and Whelan 1997 . These regulatory sequences are referred to as tissue-specific promoters (TSPs) {Binley, 1999 #283; Vile, Sunassee et al. 1998; Vassaux 1999). Introduced

genes, under the control of a TSP, are preferentially expressed in tissues that activate the TSP (Hart 1996).
Transductional targeting involves the chemical or genetic modification of adenoviruses, redirecting its tropism from the native receptor, to a new one preferentially expressed on target cells. An ideal retargeting strategy would involve blocking binding to CAR while introducing a new tropism to a tumor associated receptor or cell-surface marker. Recent advances in the understanding of adenovirus structure and biology have led to many significant achievements in these areas (Russell 2000; Volpers and Kochanek 2004). This lead to incorporation of various peptide ligands into different locations of the fiber, hexon or penton base, as well as precice point mutations in the fiber knob domains (Volpers and Kochanek 2004).

4.2 Cancer trials with adenoviral vectors

Adenoviruses are currently the most common vector system for clinical gene therapy trials for cancer. By the end of the year 2005, more than 380 clinical trials were approved and over 200 still recruiting (Database 2006). Most were phase I or phase II, which means their primary goal is determining the safety of the agents (phase I) or their potential efficacy (phase II). Genetic immunotherapy has been the most commonly tested clinical approach. Adenoviruses may be ideal vaccination vectors (Schreiber, Kampgen et al. 1999; Tillman, de Gruijl et al. 1999; Mincheff, Tchakarov et al. 2000; Stephenson 2001; Shiver, Fu et al. 2002), since they are strongly immunogenic and combine both safety and efficacy.
In addition to genetic immunotherapy, cancer trials involving adenoviral vectors can be divided in three main groups: a) suicide gene therapy, b) gene replacement, and c) receptor targeting. Suicide gene therapy typically features introduction of a prodrug-converting enzyme. Phase I trials have been performed for the treatment of glioma (Eck, Alavi et al. 1996; Sandmair, Loimas et al. 2000; Trask, Trask et al. 2000), ovarian cancer (Alvarez and Curiel 1997; Alvarez, Gomez-Navarro et al. 2000; Hasenburg, Tong et al. 2000), prostate cancer (Herman, Adler et al. 1999; Koeneman, Kao et al. 2000) and mesothelioma (Sterman, Treat et al. 1998). Interestingly, when glioma patients were resected and randomized into Ad-HSVtk/ganciclovir, retrovirus-HSVtk/ganciclovir or control groups, overall survival was significantly improved in the Ad-HSVtk/ganciclovir group (Sandmair, Loimas et al. 2000). Another randomized study with 36 glioma patients resulted as well in a significant increase for survival in patients treated with local AdvHSV-tk injections followed by systemical ganciclovir compaired to controls (Immonen, Vapalahti et al. 2004). A phase III study here is in progress.
Several trials combine suicide gene therapy with more common treatment options. Ten patients with recurrent ovarian cancer underwent secondary debulking followed first by intraperitoneal suicide gene therapy with an Ad coding for HSVtk and then intravenous ganciclovir administration and topotecan chemotherapy. When the study was published, three out of ten patients were still alive with a follow-up between 30 and 31 months (Hasenburg, Tong et al. 2001). In another phase I/II study, adenoviral suicide gene therapy with or without hormonal treatment was combined with radiotherapy for prostate cancer. The aim of this study was the expansion of the therapeutic index of radiotherapy (Teh, Aguilar-Cordova et al. 2001). No additional toxicity was noted from the combination and safety of the approach could be demonstrated. Two years after treatment, no significant long-term toxicity was detected. In the group with low-risk patients, negative biopsies and lack of metastases was seen at 21 months (Aguilar LK 2002).
With regard to gene replacement strategies, p53 has been a major target in phase I and II trials (Roth 1996; Roth, Swisher et al. 1998; Schuler, Rochlitz et al. 1998; Kauczor, Schuler et al.

1999; Swisher, Roth et al. 1999; Atencio, Warren et al. 2001; Buller, Shahin et al. 2001; Hao, Rowinsky et al. 2001; Muller, Coleman et al. 2001; Pagliaro, Keyhani et al. 2001; Pisters, McDonnell et al. 2001). Perhaps the most encouraging results were seen when Ad was given intratumorally in combination with chemotherapy (Schuler, Herrmann et al. 2001). The Chinese gouvernment approved in 2003 a recombinant Ad-p53 ("Gendicine") for clinical use (Patil, Rhodes et al. 2005; Peng 2005).
Another tumor suppressor gene used in a phase I study is mda-7. Ten patients with solid tumors were injected intratumorally followed by excision of the lesions, which allowed demonstration of transgene expression (Cunningham C 2002). Receptor targeting has been endeavored mainly for ovarian cancer with erbB2 as the target (Alvarez and Curiel 1997; Alvarez, Barnes et al. 2000). None of these studies showed dose-limiting side-effects, even with a viral dose of $7.5x10^{13}$ VP daily for five days (Buller, Shahin et al. 2001). Therefore, it can be concluded that cancer gene therapy with replication deficient adenoviral vectors is safe and although evidence of gene transfer in general has been variable, in some cases there is evidence of efficacy.

5.1 Transcriptional targeting
A promoter is the component of the genetic transcriptional unit that is involved in binding of the RNA polymerase, required for initiation of mRNA transcription. Further, the promoter is activated by transcription factors presented under tissue-specific control. Therefore, in order for a promoter to be activated in a particular tissue type, that tissue must express specific factors that recognize the promoter. A number of TSPs have been studied for cancer gene therapy, but some promoters lack sufficient activity, specificity, or both. Therefore, recent research has focused on rigorously evaluating candidate promoters with regard to these attributes for transcriptional targeting of viruses (Fig. 4A).

5.1.1 Tumor-specific promoters
One of the earliest tumor-specific promoters explored for cancer was the carcinoembryonic antigen (CEA) promoter, expressed in most gastric, pancreatic, and lung cancers (Tanaka, Kanai et al. 1996). This promoter was used to drive *HSVtk* expression and CEA-negative cell lines were resistant to ganciclovir therapy while CEA-positive cells were 1000 times more sensitive (Osaki, Tanio et al. 1994). Upon intraperitoneal injection into mice bearing CEA-expressing tumors, significant regression could be noted (Osaki, Tanio et al. 1994). Importantly, a significant by-stander effect was reported (Tanaka, Kanai et al. 1996; Tanaka, Kanai et al. 1997). An Ad carrying either *lacZ* or *CD* under the CEA promoter showed specific expression in tumor xenografts and was able to increase survival time (Lan, Kanai et al. 1997). Also, intravascular administration of an Ad employing the CEA promoter showed little toxicity in the normal liver (Brand, Loser et al. 1998). For possible treatment of hepatomas, the promoter of the alpha-fetoprotein (AFP) has been investigated. When an Ad employing this promoter was injected subcutaneously into hepatomas *in vivo*, tumor regression was noted (Kanai, Lan et al. 1997).
For treating gynecological cancers, a number of promoters have been explored. The L-plastin promoter (LP-P) was used to transcriptionally control the expression of *lacZ* in ovarian and breast cancer cell lines, and was compared to the ubiquitously expressed cytomegalovirus (CMV) promoter (Chung, Schwartz et al. 1999). Expression was observed in tumor cell lines and ascites samples with both promoters, but little activity was seen in normal human skin fibroblasts and normal peritoneum with the LP-P. Another report on LP-P showed specific expression of *lacZ* and *CD* in ovarian and bladder cancer cell lines when compared to the CMV promoter (Peng, Won et al. 2001). Over three-quarters of human epithelial ovarian

carcinomas express the DF3 protein, while normal peritoneal mesothelium does not (Friedman, Hayes et al. 1986). The DF3 promoter showed ovarian cancer-specific activity when driving the expression of *BAX in vitro* (Tai, Strobel et al. 1999). Upon intraperitoneal injection into ovarian tumor bearing nude mice, *BAX* expression was most prominent in tumor tissue and greater than 99% eradication of tumor explants was reported.
The cyclooxygenase-2 (Cox-2) promoter has also been investigated in ovarian tumor cell lines, along with the midkine (MK) promoter. Both promoters were activated in a panel of ovarian cancer cell lines, as well as, ovarian primary tumor cells, with a reduced level of activity in normal primary mesothelium and liver (Casado, Gomez-Navarro et al. 2001).
The Cox-2 promoter has also been explored in the context of gastric carcinomas. The activity profile of the promoter correlated to the Cox-2 RNA status of gastric carcinoma cell lines, and upon intravascular injection, liver expression with the Cox-2 promoter was lower than with the CMV promoter. The promoter was sufficiently active to cause tumor cell killing when driving *HSVtk* in Cox-2 positive cell lines but not negative lines. *In vivo* administration resulted in less liver toxicity with the Cox-2 promoter versus the CMV promoter (Yamamoto, Alemany et al. 2001).
The secretory leukoprotease inhibitor (SLPI) gene is expressed in several different carcinomas, including ovarian cancer. Its expression in normal organs, such as the liver, is low (Abe, Tominaga et al. 1997). Therefore, the SLPI promoter was utilized to drive transgene expression in ovarian cancer cell lines and primary tumor cells isolated from patient samples (Barker S.D. 2002). The promoter was activated in both cell lines and primary tumor cells in an Ad context *in vitro*. A murine orthotopic model of peritoneally disseminated ovarian cancer was used to demonstrate high tumor gene expression versus low liver expression with the SLPI promoter, and that Ad-delivered *HSVtk* under the control of the SLPI promoter is able to increase survival in combination with ganciclovir (Barker S.D. 2002).Further TSPs that have been studied with promising preclinical results include tyrosinase, ERBB2, surfactant protein B, proopiomelanocortin and flt-1 (Ring, Harris et al. 1996; Siders, Halloran et al. 1996; Strayer, Guttentag et al. 1998; Lee, Martinson et al. 2001).

5.1.2 Tumor vasculature-specific promoters

Targeting the endothelium of tumors may be amenable to gene therapy. This tissue is commonly independent of tumor type and more easily accessible to intravascular vector administration. Also, endothelial cells (EC) are not malignant and thus are less sensitive to selection pressure and rarely gain resistance to treatment (Nettelbeck, Jerome et al. 2000). E-selectin expression is minimal in normal blood vessels but high in the capillaries of tumors and the promoter was used for driving gene expression in an Ad. Upon infection, EC cell lines expressed high levels of reporter gene expression, while non-EC cell lines showed low expression. The addition of TNF-alpha, an inducer of the promoter, further increased the E-selectin's activity (Walton, Wang et al. 1998). The murine preproendothelin-1 (PPE-1) promoter was also used as a TSP for adenoviral-mediated delivery to EC cells. Systemic administration to lung tumor-bearing mice resulted in gene expression in the new vasculature of primary tumors (Varda-Bloom, Shaish et al. 2001).

5.1.3 Treatment responsive promoters

Another strategy for cancer gene therapy involves restricting gene expression with a conventional form of treatment, such as chemotherapy or radiation. For example, the early growth response gene 1 (EGR-1) promoter, which is radiation inducible, has been used as a TSP for the specific expression of *lacZ* and *HSVtk* in glioma and hepatocellular carcinoma

cells. Radiation-induced transcription of EGR-1 in these cells was accomplished with relatively low doses (Manome, Kunieda et al. 1998; Katabi, Chan et al. 1999).
A combination of radiation with a radiation induced adenoviral gene therapy is under development for cervical cancer models and resulted in first promossing results (Rein 2005).
TSPs have the potential to decrease the toxicity of cancer gene therapy and represent a powerful tool for the specific targeting of transgene expression to neoplastic cells. However, they do not increase the efficacy of Ad infection. By combining transcriptional targeting with infectivity enhancement, improved vectors can be developed.

5.2 Transductional targeting

Transductional targeting strategies (Fig. 4B) have the potential to increase gene transfer to target tissues and reduce sequestration by non-target tissues. There are two primary means of transductional targeting: a) genetic and b) physical. These are sometimes referred to as one-component and two-component targeting, respectively. The former involves the genetic modification of adenoviruses to incorporate ligands, which recognize specific cellular receptors, and/or block native receptor binding.

5.2.1 Genetic transductional targeting

Several areas exist in adenoviruses that are amenable for genetic insertion of ligands. One of these is the HI loop of the fiber, which was used as an insertion site for an integrin binding RGD-4C motif (Dmitriev, Krasnykh et al. 1998; Krasnykh, Dmitriev et al. 1998). RGDTKSSTR is an RGD-4C modified adenoviral vector containing *HSVtk* for molecular chemotherapy and the human somatostatin receptor subtype-2 (SSTR2) gene for non-invasive imaging (Hemminki, Belousova et al. 2001). The RGD-4C modification allowed enhanced infectivity of ovarian cancer cell lines and primary ovarian tumor cells. This enhancement was also observed in the presence of malignant ascites. Further, clinical treatment was mimicked by administering RGDTKSSTR intraperitoneally, in the presence of malignant ascites from ovarian cancer patients, to mice with disseminated ovarian cancer. A significant survival advantage was seen in comparison to an isogenic non-RGD-4C virus and other controls. Importantly, the virus could be non-invasively imaged *in vivo* for more that two weeks (Hemminki, Zinn et al. 2002). This approach was undergoing clinical evaluation with ovarian cancer patients with peritoneally disseminated disease (2001). Results of this study are not published so far.
In another study, an adenovirus containing *luciferase* and the RGD-4C modification was analyzed in comparison to a virus without the modification (Kanerva, Wang et al. 2002). In stringent preclinical substrates, including primary ovarian tumor cells (in the absence and presence of neutralizing antibodies) and in a murine model of ovarian cancer, increased gene expression was observed with the RGD-modified virus.
Because adenovirus has a propensity to localize to the liver, with potential for hepatotoxicity, untargeting the liver is an important goal (Lieber, He et al. 1997; Hemminki and Alvarez 2002). In this regard, viruses were created, which lack binding to CAR and to cellular integrins since both play a significant role in liver uptake. These viruses could be shown to be effectively reduced in their liver transduction capability (Einfeld, Schroeder et al. 2001). By additional ablation of the heparin sulfate glycosaminoglycan-binding site of the fiber shaft, which is proven to be a keypart of adenoviral-5 infection besides CAR and integrins, liver transduction in vitro and in vivo could be abrogated, while transduction of target tissue was reduced as well but to a much smaller degree (Bayo-Puxan, Cascallo et al. 2006).

5.2.2 Fiber chimerism

Not all adenoviral serotypes bind CAR and therefore have a different tropism. Fiber chimerism involves replacing the fiber or knob domain with a knob of a different serotype (Krasnykh, Mikheeva et al. 1996; Von Seggern, Huang et al. 2000; Kanerva, Zinn et al. 2003; Raki, Kanerva et al. 2005). For example, the Ad3 serotype has a yet unidentified receptor. Whereas first CD46 was reported to be the Ad3 receptor (Sirena, Lilienfeld et al. 2004), recent publications suggest that CD46 is a receptor for the B-group Ads but not for Ad3 (Marttila, Persson et al. 2005; Tuve, Wang et al. 2006).

Nevertheless, a chimeric Ad5/3 vector increased gene transfer by up to 291-fold in ovarian cancer cell lines and primary tumor cells (Kanerva, Mikheeva et al. 2002). Importantly, the biodistribution and murine toxicity of the chimeric and RGD modified viruses was not significantly different from the Ad5 based vectors, which have proven safe in clinical trials.

Further, adenoviruses with polylysine motifs in the fiber C-terminus, enabling CAR-independent binding to heparan sulfate proteoglycans (HSPG) have been constructed. Heparan sulfate proteoglycans represent a subgroup of adenovirus receptors, especially expressed on tumor cells. Therefore, these viruses have showed increased infectivity for tumor cells (Kangasniemi, Kiviluoto et al. 2006; Ranki, Kanerva et al. 2006; Sarkioja, Kanerva et al. 2006).

5.2.3 Physical transductional targeting

Physical targeting involves complexing adenovirus with a bispecific molecule, which both blocks binding to CAR and redirects the virus to new specific receptors. A major advantage to using this form of targeting is the abundance of antibodies and ligands that can be utilized. For example, basic fibroblast growth factor (FGF2) has been conjugated to the Fab fragment of an anti-knob antibody. This Fab-FGF2 conjugate was able to increase transgene expression by more than 9-fold in ovarian cancer cell lines, and upon intraperitoneal injection of HSVtk viruses into tumor-bearing mice, survival was prolonged from 36 to 44 days (Rancourt, Rogers et al. 1998). In addition decreased hepatic toxicity was demonstrated (Gu, Gonzalez et al. 1999). This strategy resulted in increased survival also in a melanoma xenograft mouse model (Gu, Gonzalez et al. 1999). Other Fab-ligand conjugates have been employed in a similar manner with promising results (Douglas, Rogers et al. 1996; Goldman, Rogers et al. 1997; Haisma, Pinedo et al. 1999; Reynolds, Zinn et al. 2000).

Another transductional targeting concept is the sCAR-ligand conjugat, utilizing the secretory ectodomain of CAR fused with a targeting ligand. For example, epidermal growth factor (EGF) has been conjugated to sCAR and used to target adenoviruses to cancer cells that overexpress the EGF receptor (Dmitriev, Kashentseva et al. 2000; Wesseling, Bosma et al. 2001). A dose-dependent increase of luciferase expression was reported in cell lines with both a replication-defective and a oncolytic adenovirus (Hemminki, Dmitriev et al. 2001). When infected cells were injected subcutaneously, only 1% of targeted adenoviral-infected cells were needed to inhibit tumor growth and only 5% were needed to heal tumors. sCAR has also been fused to a single-chain antibody specific for the c-erbB-2 oncoprotein. Again, significant increases in gene transfer were observed (Kashentseva, Seki et al. 2002).

Although two-component targeting has shown promising results for retargeting adenovirus to new receptors, it may present some disadvantages. Two-component gene delivery systems have more complex pharmacodynamics and -kinetics, and their stability in humans has not yet been studied. Therefore, one-component systems may be more easily applicable to human cancer gene therapy trials.

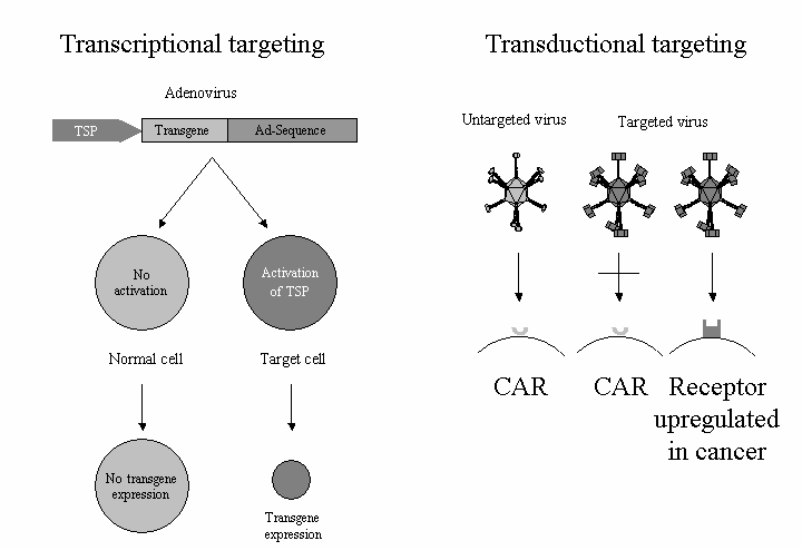

Fig. 4. A) Transcriptional targeting: a tissue specific promoter is placed in front of the transgene or a gene essential for viral replication. Only cells expressing the tissue specific promoter can activate transcription of transgene (non-replicative adenoviruses) or viral replication (replicative adenoviruses). B) Transductional targeting: The fiber knob of the adenovirus, responsible for determining the tropism, is replaced or blocked with a retargeting moiety. This redirects the virus to a cancer associated receptor.

6.1 Conditionally replicating adenoviruses (CRAds) as oncolytic agents

Although non-replicating first generation adenoviruses have provided high *in vitro* and *in vivo* transduction rates and good safety data, clinical trials have suggested, that the antitumor effect may not be sufficient in a single treatment approach (Glasgow, Bauerschmitz et al. 2004). Although tumor targeting and infectivity enhancement have improved preclinical results dramatically, it is possible that clinical application of non-replicating agents may require multiple rounds of re-administration. Viruses that replicate and spread specifically inside the tumor have been suggested as a way to improve tumor penetration with an additional benefit of local amplification of effect. To this end, CRAds have been developed. These viruses are genetically modified to take advantage of tumor specific changes that allow preferential replication of the virus in target cells (Alemany, Balague et al. 2000; Curiel 2000; Curiel 2000; Gomez-Navarro and Curiel 2000; Heise, Hermiston et al. 2000; Balague, Noya et al. 2001). The viral replication cycle causes oncolysis of the cell, resulting in the release of the newly generated virions and subsequent infection of neighboring cells (Fig. 5). Thus, the anti-tumor effect is not delivered with a transgene but by the actual replication of the virus. In theory, the oncolytic process continues as long as target cells for the virus persist.

There are two main ways to control viral replication. One is the control of replication regulators, such as the viral early gene E1, with TSPs, resulting in Type I CRAds. The other method involves introduction of deletions in the viral genome (Type II CRAd) that require specific cellular factors to compensate the effects of these deletions (Heise, Sampson-

Johannes et al. 1997; Fueyo, Gomez-Manzano et al. 2000). Further, both approaches can be combined with the potential for increased specificity (Nettelbeck, Rivera et al. 2002).
A promoter is a DNA sequence that enables a gene to be transcribed and can work in concert with other regulatory regions (e.g. enhancers, silencers, insulators) to direct the level of transcription of a given gene. In case of CRAds, the transcription level of genes critical for the replication process is controlled, allowing replication of the virus only in those cells, where the promoter element is active.
Various promoters have been used to control viral replication (Rodriguez, Schuur et al. 1997; Hallenbeck, Chang et al. 1999; Yu, Sakamoto et al. 1999; Hernandez-Alcoceba, Pihalja et al. 2000; Kurihara, Brough et al. 2000; Bauerschmitz, Guse et al. 2006; Takayama, Reynolds et al. 2006; Zhu, Chen et al. 2006). Typically, the TSP is placed to control expression of E1A, the crucial regulator of Ad replication. PSA and kallikrein-2 have been used in the context of prostate cancer and AFP has been used for hepatoma (Rodriguez, Schuur et al. 1997; Gu, Gonzalez et al. 1999; Hallenbeck, Chang et al. 1999). When the DF3/MUC1 promoter was used to drive expression of E1A in breast cancer cells, replication at levels comparable to wild-type Ad was seen, while in negative cell lines, replication was decreased. A single intratumoral injection of this TSP-controlled CRAd resulted in significant reduction of tumor burden (Kurihara, Brough et al. 2000). For the treatment of pediatric solid tumors, a CRAd featuring the midkine (MK) promoter was utilized. This CRAd achieved specific and high levels of replication in MK-positive cell lines and was able to induce tumor cell killing *in vitro* (Adachi, Reynolds et al. 2001). To further increase the oncolytic effect, transgenes for cytokines or prodrug-activating enzymes have been included (Freytag, Rogulski et al. 1998; Wildner, Morris et al. 1999; Kurihara, Brough et al. 2000). The latter approach could also allow abrogation of virus replication in case of toxicity.
Heretofore, three to four approaches have been utilized for creation of deletion type CRAds. Here, the replication of the virus is limited by insertion of a deletion in a viral replication gene, which blocks replication in normal cells, but can be overcome by mutated cells, allowing replication exclusively in those cells.
The first one was ONYX-015, which has two mutations in the gene coding for the E1B 55-kd protein (Cohen and Rudin 2001; McCormick 2003). The purpose of this protein is binding and inactivation of p53 in infected cells, for induction of S-phase, required for virus replication (Bischoff, Kirn et al. 1996; Heise, Sampson-Johannes et al. 1997; Heise, Williams et al. 1999; Rogulski, Freytag et al. 2000). Thus, this virus might have preference for replication in cells with mutated p53, a common feature in human tumors (Bischoff, Kirn et al. 1996), although alternative mechanisms have also been suggested (Macrae, Neve et al. 2005), and selectivity in general has been disputed (Edwards, Dix et al. 2002; Wadler, Yu et al. 2003). Nevertheless, initial studies have suggested that this agent replicates more effectively in tumor than in normal cells (Heise, Sampson-Johannes et al. 1997; Rothmann, Hengstermann et al. 1998; Hay, Shapiro et al. 1999; Alemany, Balague et al. 2000; Dix, Edwards et al. 2001). Unfortunately, the function of E1B55kD is not limited to p53 binding (Dix, Edwards et al. 2001), which causes inefficient replication of the virus compared with the wild type adenovirus (Barker and Berk 1987; Bischoff, Kirn et al. 1996; Hay, Shapiro et al. 1999). In addition, recent studies have suggested replication of ONYX-015 in nontarget normal tissue (Wadler S 2002; Cherubini, Petouchoff et al. 2006).
The second group of deletion mutants have a 24 bp deletion in the constant region 2 (CR2) of the E1A gene (Heise, Sampson-Johannes et al. 1997; Fueyo, Gomez-Manzano et al. 2000; Suzuki, Fueyo et al. 2001). This domain of the E1A protein is responsible for binding the retinoblastoma tumor suppressor/cell cycle regulator protein (Rb), required for effective replication (Russell 2000). Viruses with this type of deletion have reduced ability to overcome

the G1-S checkpoint and replicate efficiently only in cells where this interaction is not necessary, e.g. tumor cells defective in the *Rb-p16* pathway (Fueyo, Gomez-Manzano et al. 2000; Heise, Hermiston et al. 2000). Appropriately, this pathway seems to be inactive in almost all human tumors (Sherr 1996). It has been shown that replication of CR2-deleted viruses is attenuated in non-proliferating normal cells (Fueyo, Gomez-Manzano et al. 2000; Heise, Hermiston et al. 2000). Interestingly, abrogation of replication was also demonstrated when Rb was re-introduced into otherwise permissive cells (Fueyo, Gomez-Manzano et al. 2000).
Adenoviruses with mutations in CR1 and CR2 domains of E1A were found to replicate selectively in tumor cells expressing human papillomavirus E6 and E7 oncoproteins (Balague, Noya et al. 2001). Further, CRADs featuring an additional mutation in CR3, the binding site for p300, cell cycle regulator, have been described (Doronin, Toth et al. 2000). These viruses were further modified by replacing the natural promoter of E4 by a TSP (Doronin, Kuppuswamy et al. 2001).

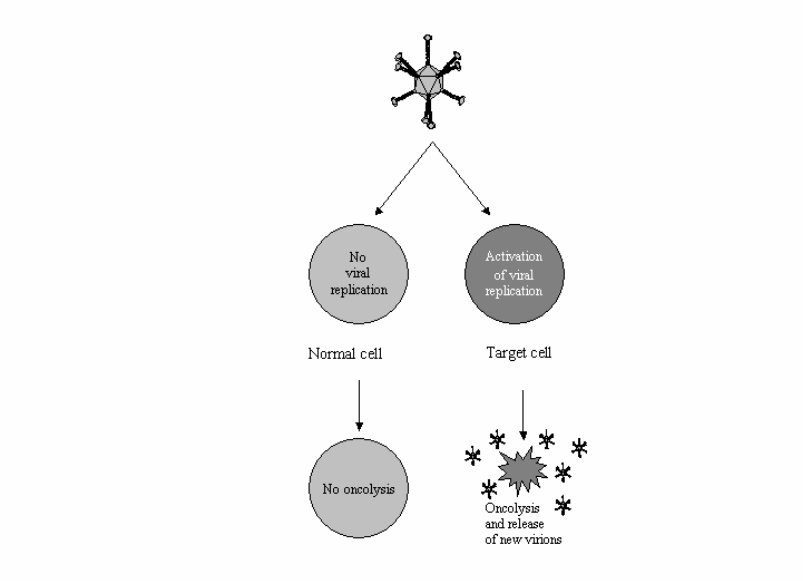

Fig. 5. Conditionally replicating adenoviruses are restricted in their replication to specific factors of their target (cancer) cells. Replication results in oncolytic death and release of virions to surrounding cells.

6.2 Targeted conditionally replicating adenoviruses

Non-targeted CRAds infect cells mostly based on their CAR-level, which may be highly variable in clinical cancers. Nevertheless, even such first generation CRAds have shown evidence of clinical utility (Hemminki and Alvarez 2002). These initial successes suggest that if efficiency of infection and specificity of replication of the agents could be enhanced, further improvements in clinical efficacy could be gained. This is corroborated by demonstration of the close association between infectivity and oncolytic potency (Shinoura, Yoshida et al. 1999; Douglas, Kim et al. 2001; Hemminki, Dmitriev et al. 2001). Consequently, infectivity enhanced CRAds have been constructed, with impressive preclinical efficacy. Ad5-Δ24RGD features an RGD-4C modification of the fiber (Suzuki, Fueyo et al. 2001; Lamfers, Grill et al.

2002), and displays similar oncolytic potency to wild type virus in ovarian cancer cells. Further, this virus is able to replicate in ovarian cancer primary cell spheroids and results in significantly prolonged survival in an aggressive orthotopic ovarian cancer model (Bauerschmitz, Lam et al. 2002). These developments have led to clinical trial protocols, where glioma and ovarian cancer patients will be treated with the Ad5-Δ24RGD virus (Kanerva and Hemminki 2005).
As another approach, fiber-chimerism was used to increase infectivity of CRAds for tumor cells. These viruses contain chimeric fibers with the tail and shaft from Ad5 and the knob domain of Ad3 (Kanerva, Mikheeva et al. 2002; Kanerva, Wang et al. 2002; Kanerva, Zinn et al. 2003; Breidenbach, Rein et al. 2004) and Ad5/3Δ24 showed increased oncolytic potency and preferential replication within tumor tissue. Here as well a protocol for a clinical trial is approved and this trial is supposed to open shortly. Besides the 5/3 chimera, chimeras Ad5/11 (Stecher, Shayakhmetov et al. 2001; Yu, Takenobu et al. 2005) and Ad5/35 (DiPaolo, Ni et al. 2006; Ni S 2006) are investigated (Stone and Lieber 2006).
Recent publications use double or even triple targeted CRAds to increase specificity while oncolytic potency is maintained (Nettelbeck, Rivera et al. 2002; Kanerva, Bauerschmitz et al. 2004; Bauerschmitz, Guse et al. 2006). For example, the combination of a tumor specific promoter cox2L, E1A transcomplementation and 5/3-fiber chimerism resulted in the CRAd Ad5/3cox2Ld24, showing highly selective replication in tumor tissue without reducing potency (Bauerschmitz, Guse et al. 2006).
A major problem in assessing CRAd efficacy and safety preclinically, is the lack of an appropriate animal model. Human serotype Ads or CRAds do not replicate productively in commonly used mouse or other animal models. Therefore, meaningful safety data is difficult to obtain, and efficacy data may be skewed due to deficient immune responses in xenograft models. To this end, several new models were investigated up to now, and cotton rats (Toth, Spencer et al. 2005), Syrian hamsters (Thomas, Spencer et al. 2006) or swine (Jogler, Hoffmann et al. 2006) showed at least to a certain degree replication of human Ads.

6.3 Cancer trials with CRAds

The first cancer trials with replicating adenoviruses were done shortly after the virus was detected in the 1950s. Various serotypes of wild type adenoviruses were applied intratumorally, intravenously & intraarterially in combination or intravenously into patients with cervical carcinoma. The effect of immune suppression was also investigated. The overall response rate, measured as formation of necrotic areas, was 65% (Smith, Huebner et al. 1956). The authors do not describe severe side effects, but relapse was common. The first trial with a CRAds started nearly 50 years later and was predicated on the development of viral agents where replication was more selective for the target tissue (Table I).
The first CRAd used in clinical trials was the ONYX-015 virus. In a limited number of biopsy specimens, replication of the agent in tumor cells was demonstrated and the safety data was excellent (Ganly, Eckhardt et al. 2000; Nemunaitis, Ganly et al. 2000). Since oncolysis can be synergistic to the effects of radiation or chemotherapy and side effects are theoretically non-overlapping (Heise, Sampson-Johannes et al. 1997; Rogulski, Freytag et al. 2000), it seems obvious to combine CRAd approaches with these treatments. Recent clinical studies show promising results and impressive safety data (Khuri, Nemunaitis et al. 2000; Lamont, Nemunaitis et al. 2000; Nemunaitis, Ganly et al. 2000).
Exciting data was obtained in a phase II study utilizing a combination of intratumorally injected ONYX-015 with simultaneous cisplatin and 5-fluorouracil chemotherapy in 30 patients with advanced stage head and neck cancer (Khuri, Nemunaitis et al. 2000). Eighty-three percent of the tumors responded; in 63% the response was objective with more than

50% tumor size reduction. Partial response was shown in 36%, and 27% showed a complete response. This is of interest since head and neck cancer is often refractory to available treatments. Though patients had several tumors, only one was chosen for viral injection. In follow-up, the non-injected tumors relapsed more frequently than the injected tumors. Similar data was obtained in another study with a similar approach (Lamont, Nemunaitis et al. 2000). A phase III trial with ONYX-015 was attempted in the US in 2002 (Lamont, Nemunaitis et al. 2000), but it appears that production issues prevented initiation of this trial. Instead, a phase III trial was performed in China with a closely related virus, H101 (Xia, Chang et al. 2004). Adding virus to chemotherapy increased response rates from 39.6 to 78.8 %, while evidently no increase in severe toxicity was seen. This is the only randomized trial with oncolytic viruses performed thus far..
Another CRAd used in clinical studies is CV-706, where viral replication is under the control of the PSA promoter (DeWeese, Drew et al. 2001). In a phase I study with 20 patients, good safety data could be obtained. In addition, preliminary evidence of viral replication and antitumor effect could be observed.

7. Model systems – primary cells and spheroids

The preclinical development of novel approaches with replication competent viruses is limited by assay substrates. As a result of adaptation to growth in vitro, established cell lines may have undergone geno- and phenotypic changes, resulting in a disconnect between data obtained from cell lines and clinical specimens. The translational approach from the bench to the clinic would benefit from models that reproduce the patient phenotype as closely as possible. In this regard, the isolation of pure cancer cells from patient samples is an attractive concept. A method for the isolation of primary ovarian tumor cells from the ascitic fluid of patients diagnosed with ovarian adenocarcinoma has been described. Populations of up to 96% purity have been isolated in this manner (Barker, Casado et al. 2001). Unfortunately, primary tumor cells are difficult to analyze *ex vivo* for virus replication due to their limited viability in culture (Casado 2001), which is approximately seven days and too short for typical assays. In addition, monolayers may not reflect virus dissemination characteristics appropriately as most human solid tumors are three dimensional. Therefore, model systems based on three-dimensional aggregates or spheroids of unpassaged and purified ovarian cancer cells have been developed to overcome these obstacles (Lam, Kanerva et al. 2001; Kanerva, Zinn et al. 2003; van Beusechem, Mastenbroek et al. 2003; Rein, Breidenbach et al. 2005). Spheroids were viable for more than four weeks and allowed quantitation of CRAd replication.

8. Future prospects

With increasing understanding of the molecular reasons for cancer, gene therapy has emerged as a logical potential therapeutic option. Following initial optimism and subsequent disappointment, rigorous preclinical and basic research is now beginning to result in clinically feasible approaches. Considering the immunogenicity of adenoviruses, while useful for mounting an immune response to the tumor, has the potential for severe or even fatal toxicity when large doses are administered (Raper, Yudkoff et al. 2002), it is important to note that safety has been never a limiting issue in cancer trials (Kanerva and Hemminki 2005; Vattemi and Claudio 2006). Further, there are exciting preliminary results suggesting efficacy (Khuri, Nemunaitis et al. 2000; Sandmair, Loimas et al. 2000; Schuler, Herrmann et al. 2001; Immonen, Vapalahti et al. 2004; Xia, Chang et al. 2004; Peng 2005).
Importantly, the feasibility of gene therapy for correction of disease phenotypes has been demonsrated in other fields of medicine (Isner and Asahara 1999; Cavazzana-Calvo, Hacein-

Bey et al. 2000; Kay, Manno et al. 2000; Hacein-Bey-Abina, Le Deist et al. 2002). What these successes shared in common is the rational approach investigators took for incrementally developing their gene delivery tools. Thus, the clinical breakthroughs were based on advances in vector development. It remains to be seen if consistent improvements in cancer gene therapy reagents can eventually deliver similar clinical success. In that regard, the key issue remains improving tumor transduction. Fortunately, we have increasingly powerful tools to address this problem, including replication competent systems, infectivity enhancement and targeting strategies.

One other obstacle remains as the limited possibility to interact with viral replication in vivo after administration. Thus, treatment for viral side effects was only symptomatic so far. With chlorpromazine and apigenin replication of adenoviruses could be desreased in vitro and in vivo, theretofore possible viral toxicity in a clinical trial treated causally (Kanerva, in press).

9. Conclusions

Current treatment options are limited for many types of human carcinomas and especially therapy of advanced disease is often palliative. In recent decades, we have seen improvements in the treatment of patients with early disease, due to aggressive adjuvant chemotherapy regimens, refined radiotherapy and advanced operative techniques. However, benefit for advanced stage cancer patients have been less dramatic. Also, the toxicity of many current treatments remains significant. Thus, there is a need for new and innovative therapeutic approaches, which may be able to overcome these limitations. Although gene therapy has proven to be a potential candidate, and preliminary evidence of clinical safety and efficacy exist, utility, there are still obstacles to overcome. Considering the synergistic or additive effect many gene therapy approaches have with existing treatments such as radiation or chemotherapy, it is likely that the first routine clinical applications will be combination treatments. Further, it is noteworthy that the side effect profile of adenoviral treatments seems to have little or no overlap with radiation or chemotherapy.

Table 1. Cancer trials with conditionally replicating adenoviruses[a]

Approach	Phase	Patients	Max. dose[f]	Route[c]	Disease[d]	Ref.
wild type Ad (various serotypes)	nd[b]	30	nd[h]	it, ia, iv	cervical ca.	(Smith, Huebner et al. 1956)
ONYX-015	I	22	$1x10^{11}$ PFU	it	SCCHN	(Ganly, Eckhardt et al. 2000)
ONYX-015	I	23	$1x10^{11}$ PFU	it	pancreas ca.	(Mulvihlll, Warren et al. 2001)
ONYX-015	I	10	$2x10^{13}$ VP	iv	ca. metastatic to lung	(Nemunaitis, Cunningham et al. 2001)
ONYX-015	I	16	$1x10^{13}$ VP/d x5d	ip	ovarian ca.	(Kirn 2001)
ONYX-015	I	16	$1x10^{11}$ PFU/d x5d	ip	ovarian cancer	(Vasey, Shulman et al. 2002)
dl1520-TK-CD[e,g]	I	9	$1x10^{12}$ VP	it	prostate ca.	(Khil, Aguilar-Cordova et al. 2001)
CV706	I	20	$1x10^{13}$ VP	it	prostate ca.	(DeWeese, Drew et al. 2001; DeWeese, van der Poel et al. 2001)
ONYX-015	I-II	33	$2x10^{12}$ VP	iha	colorectal (and other GI) ca. metastatic to liver	(Reid, Galanis et al. 2001)
dl1520[e] + 5-FU (in Phase II)	I-II	16	$3x10^{11}$ PFU	it, iv, iha	hepatocell. ca. and GI ca. metastatic to liver	(Habib, Sarraf et al. 2001)
ONYX-015	II	40	$1x10^{11}$ VP/d x 10d/2wk cycle	it	SCCHN	(Nemunaitis, Ganly et al. 2000; Nemunaitis, Khuri et al. 2001)
ONYX-015 + cisplatin + 5-FU	II	37	$1x10^{10}$ PFU/d x 5d	it	SCCHN	(Khuri, Nemunaitis et al. 2000)

ONYX-015 + cisplatin + 5-FU	II	14	$1x10^{10}$ PFU/d x 5d	it	SCCHN	(Lamont, Nemunaitis et al. 2000)
ONYX-015 +/- gemcitabine	I-II	21	$2x10^{11}$ VP, 1/wk, 8 cycles	it	Pancreatic	(Hecht, Bedford et al. 2003)
ONYX-015	II	18	$2x10^{12}$ VP every two weeks	iv	Metastatic colorectal cancer	(Hamid, Varterasian et al. 2003)
CG7870	I-II	20	$1x10^{13}$ VP	it	Locally recurrent prostate Ca	(DeWeese, Arterbery et al. 2003)
H101 + cisplatin or adriamycin + 5-FU	III	160	$1.5x10^{12}$ VP/d x 5d	i.t.	SCCHN	(Xia, Chang et al. 2004)
CG7870	I	23	$6X10^{12}$ VP	i.v.	Metastatic prostate cancer	(Small, Carducci et al. 2006)
Total		**528**				

[a] An attempt was made to include most published cancer gene therapy clinical trial with oncolytic adenoviruses. For a complete listing of gene therapy trials, see (http://www4.od.nih.gov/oba/rac/clinicaltrial.htm; http://www.wiley.com/genetherapy/clinical/)

[b] **nd** = not determined

[c] **iv** = intravenous, **ia**. intra-arterial, **it** = intratumoral, **ip** = intraperitoneal, **iha** = intrahepatic artery

[d] **ca.** = cancer, **SCCHN** = squamous cell cancer of the head and neck, **GI** = gastrointestinal

[e] dl1520 is original name of ONYX-015, also known as CI-1042.

[f] refers to maximum dose administered per cycle if multiple cycles were used. Often, maximum tolerated dose was not reached. **VP** = viral particles. **PFU** = particle forming (infectious) units.

[g] **TK** = herpes simplex virus thymidine kinase, **CD** = cytosine deaminase

[h] not determined with methodology comparable to current assays

AIMS OF THE STUDY

This study was performed in order to develop new treatment modalities for gynecologic cancer patients, who have metastaciesed disease and often not benefit any more from current treatment options. Adenoviral Gene Therapy was utilized and a new generation of vectors developed.

- Specific Aim #1: To measure expression of the flt-1 promoter in different gynecologic cancer cell types and to evaluate its potential usefulness for teratocarcinoma **(I)**

- Specific Aim #2: To evaluate replication and oncolytic potency in vitro and in vivo of Ad5-Δ24RGD for treatment of ovarian cancer **(II)**

- Specific Aim #3: To evaluate oncolytic potency in vitro and in vivo of Ad5-Δ24RGD for cervical cancer, to study biodistribution and to assess infectivity and toxicity to human peripheral blood mononuclear cells **(III)**

- Specific Aim #4: To construct advanced generation triple targeted oncolytic adenoviruses featuring the cox-2 promoter, E1A transcomplementation and serotype chimerism, to measure their oncolytic potency for ovarian cancer cells in vitro and in vivo and to study replication of the agents in vivo **(IV)**

MATERIALS AND METHODS

Detailed description of the used methodology can be found in the original publications.

1. Cell lines and primary cells (I-IV)

Table 2. The list of human cell lines used in this study

Cell line	Description	Used in
293	Transformed embryonic kidney cells	I, II, III, IV
911	Transformed embryonic retinoblasts	IV
A549	Lung adenocarcinoma	II, III, IV
ES-2	Ovarian adenocarcinoma	IV
Hey	Ovarian adenocarcinoma	IV
OV-4	Ovarian adenocarcinoma	IV
SKOV-3.ip1	Ovarian adenocarcinoma	IV
NCCIT	Testicular teratocarcinoma	I
NTERA-2	Testicular teratocarcinoma	I
PA-1	Ovarian teratocarcinoma	I
F9	Murine ovarian teratocarcinoma	I
AU565	Mammary gland adenocarcinoma	I
GI-101A	Mammary gland adenocarcinoma	I
ZR-75-1	Ductal breast cancer	I
C33A	Squamous cervical carcinoma	III
Caski	Squamous cervical carcinoma	III
Hela	Cervical adenocarcinoma	III
SiHa	Squamous cervical carcinoma	III
BEAS-2B	Lung epithelial cells	I
BT474	Mammary adenocarcinoma	IV
FHS173WE	Fibroblast	IV
HMEC	Mammalian endothelial cells	IV
HUVEC	Umbilical vein endothelial cells	III
nHEPs	Clonetics™ Human Hepatocytes	IV

Cell lines were subcultered according to the providers recommendations and not used with passage numbers above thirty.

Primary ovarian adenocarcinoma cells were purified by an immunomagnetic-based method from malignant ascites fluid samples from patients undergoing a procedure for ovarian cancer at the University of Alabama at Birmingham Hospital (Barker, Casado et al. 2001). Briefly, ovarian cancer cells were bound with a murine anti-TAG-72-antibody (CC-49, a generous gift from Dr. J. Schlom, National Institute of Health, Bethesda, MD) and then collected with magnetic beads coated with anti-mouse-IgG (Pan Mouse IgG Dynabead, Dynal AS, Oslo, Normway) (II).

Analysis and creation of spheroids from primary ovarian carcinoma cells (II) is detailed elsewhere (Lam, Kanerva et al. 2001). Briefly, purified unpassaged cells were incubated

overnight in a 3% agar coated flask on a rocker to form spheroids, *ie.* three-dimensional clusters of cells. The spheroids were resuspended and used for experiments.
Ficoll-Hypaque separated normal donor peripheral blood mononuclear cells (PBMC) were obtained from fresh citrated blood. Blood was mixed with PBS (Mediatech, Herndon, VA) and underlayered with Histopaque®-1077 (Sigma Diagnostics, St. Louis, MO) for gradient centrifugation. After two washing cycles with PBS, viable PBMCs were counted with Trypan Blue staining (Mediatech) and cells were dispensed into round bottom 96 well plates for incubation in RPMI 1640 medium containing 2 mM L-glutamine (Mediatech) and 10% FBS (HyClone).

2. Adenovirus vectors and replicating adenoviruses (I-IV)

Table 3. The list of adenovirus vectors used in this study

Virus	Description	Used in
Ad5flt-1luc	E1-deleted, a luc gene under the flt-1 promoter in place of E1	I
AdCMVluc1	E1-deleted, a luc gene under the CMV promoter in place of E1	I
Ad5flt-1LacZ	E1-deleted, a lacZ gene under the flt-1 promoter in place of E1	I
Ad5CMVLacZ	E1-deleted, a lacZ gene under the CMV promoter in place of E1	I
Ad5lucRGD	E1-deleted, a luc gene under the CMV promoter in place of E1, RGD-4C modification in the HI loop of the knob domain	II, III
Ad5/3luc1	E1-deleted, a luc gene under the CMV promoter in place of E1, chimeric fiber with the tail and shaft from Ad5 and the knob domain of Ad3	IV

Table 4. The list of CRAds used in this study

Virus	Description	Used in
Adwt300	wildtype adenovirus	III, IV
Ad5RGD	RGD-4C modification in the HI loop of the knob domain	II
Ad5-Δ24RGD	24-bp deletion in CR2 of E1A, RGD-4C modification in the HI loop of the knob domain	II, III
Ad5/3-Δ24	24-bp deletion in CR2 of E1A, chimeric fiber with the tail and shaft from Ad5 and the knob domain of Ad3	IV
Ad5/3cox2LE1	E1A under cox2L promoter, chimeric fiber with the tail and shaft from Ad5 and the knob domain of Ad3	IV
Ad5/3cox2Ld24	24-bp deletion in CR2 of E1A under the cox2L promoter, chimeric fiber with the tail and shaft from Ad5 and the knob domain of Ad3	IV
Ad5/3cox2Ld2d24	24-bp deletion in CR2 with additional 6-bp deletion of E1A under the cox2L promoter, chimeric fiber with the tail and shaft from Ad5 and the knob domain of Ad3	IV

Ad5/3cox2ME1	E1A under the cox2M promoter, chimeric fiber with the tail and shaft from Ad5 and the knob domain of Ad3	IV
Ad5/3cox2Md24	24-bp deletion in CR2 of E1A under the cox2M promoter, chimeric fiber with the tail and shaft from Ad5 and the knob domain of Ad3	IV
Ad5/3cox2Md2d24	24-bp deletion in CR2 with an additional 6-bp deletion of E1A under the cox2M promoter, chimeric fiber with the tail and shaft from Ad5 and the knob domain of Ad3	IV

Table 5. The plasmids used for cloning the viruses

Plasmid	Description	Used in
pShuttle GL3Bcox-2pL	Shuttle plasmid containing cox2L promoter driving GL3B	IV
pShuttle GL3Bcox-2M	Shuttle plasmid containing cox2M promoter driving GL3B	IV
pSE1	Shuttle plasmid containing wild type E1A	IV
pSE1d24	Shuttle plasmid containing 24-bp deletion in CR2 of E1A	IV
pSE1d2d24	Shuttle plasmid containing 24-bp deletion in CR2 with additional 3-bp deletion in E1A	IV
pTU5/3	E1-deleted Ad5 genome with chimeric fiber with the tail and shaft from Ad5 and the knob domain of Ad3	IV

2.1 Construction of cox-2 CRAds (IV)

The different CRAds were constructed by digesting pShuttleGL3Bcox-2pL and pShuttleGL3Bcox-2M with KpnI and HindIII, respectively, to achieve the different cox-2 promoter sequences. pSE1, pSE1d24 and pSE1d2d24 were digested with XhoI and NotI to remove the natural E1 promoter. After blunt-ending with T4-Polymerase the two different cox2 promoter sequences were included into the different pSE shuttles. Direction of the inserted promoter was checked by PCR. The viral backbone was rescued with PacI digested pTU-5/3, which includes GFP and luciferase instead of E1. Specifically, the six different PacI digested pSEs were cotransfected with the digested pTU-5/3 into 911 cells. Plaques were picked 7 to 20 days after infection and checked for presence of the expected promoter-E1 combination and Ad5/3 fiber modification as well as absence of wt-E1 and GFP by PCR.

2.2 High titer production of viruses (I-IV)

Propagation of the adenovirus vectors and CRAds was performed on 293 and A549 cells, respectively. All viruses were purified on cesium chloride gradients. The VP concentration was determined at 260 nm, and standard plaque assay on 293 cells was performed to determine infectious particles.

3. In vitro experiments (I-IV)

3.1 Luciferase assay (I)

Cell lines were plated on day 1 at 25,000 cells per well on 24-well plates in 1 ml growth media (GM). On day 2, cells were infected with 5, 50 or 500 PFU/cell for 2 h in 200 µl 2% GM on a rocker. Afterwards, cells were washed once with 1 ml PBS and 1 ml GM was added per well. After 24 hours the GM was removed, cells were lysed with 200 µl lysis buffer (Reporter Lysis Buffer, Promega, Madison, WI) and freeze-thawed once. 20 µl of these samples was mixed with 100 µl of luciferase assay reagent (Reporter Lysis Buffer, Promega, Madison, WI) and measured with Berthold Lumat LB9501. Standardization was accomplished by setting the values obtained with CMV promoter as 100% for each cell line.

3.2 LacZ-staining (I)

Cell lines were plated on day 1 at 50,000 cells per well on 24-well plates in 1 ml GM. On day 2, cells were infected with 500 PFU/cell for 2 h in 200 µl 2% GM on a rocker. Afterwards, cells were washed once with 1 ml PBS and 1 ml GM was added per well. After 24 hours, the GM was removed and cells were washed twice with PBS. Cells were fixed for 15 min with 0.5% glutaraldehyde and washed again twice with PBS. Cells were stained for 2 1/2 hours with standard X-gal solution (containing 40 µl 2% X-gal, 10 µl 0.3 M potassium ferricyanide, 10 µl 0.3 M potassium ferrocyanide and 940 µl PBS per ml), washed again for 10 min with PBS and fixed a second time with 10% buffered formaline for 30 min. Pictures were taken by bright field microscopy at 10x magnification.

3.3 RT-PCR (I)

RNA of cells was extracted with RNeasy mini prep kit (Qiagen, Valencia, CA), treated with Deoxyribonuclease I (Life Technologies, Rockville, MD) for 30 min and RT-PCR of 90 ng RNA each was performed with the OneStep RT-PCR Kit (Qiagen, Valencia, CA) using the following primers: Flt-1 sense: 5'-TGC TTG AAA CCG TAG CTG G-3', Flt-1 antisense: 5'-GGT GCC AGA ACC ACT TGA TT-3'; GAPDH sense: 5'-TCC CAT CAC CAT CTT CCA-3'; GAPDH antisense: 5'-CAT CAC GCC ACA GTT TCC-3'. Preliminary serial dilution assays were used to determine the linear range of amplification for the genes under investigation.

3.4 Crystal violet assay (II)

Cells were plated at 500,000 cells per well on 6-well plates and infected with 0, 0.1, 1 or 10 VP/cell for 1 h, followed by washing. Growth medium with 5% FBS was changed every other day. On day 10 (PA-1), day 14 (Hey and SKOV3.ip1) or day 17 (OV-4), respectively, crystal violet staining was performed as described (Hemminki, Dmitriev et al. 2001).

3.5 Mitochondrial oncolysis assay (II, III, IV)

MTS assay was performed as described (Bauerschmitz, Lam et al. 2002). Briefly, with ovarian cancer cell lines, cells were plated at 15,000 cells per well on 96-well plates and infected with 0, 0.1, 1 or 10 VP/cell for 1 h in 50 µl medium (2% FBS) on a rocker. Cells were incubated in medium with 5% FBS, which was changed every other day. On day 8 (PA-1), day 15 (Hey and SKOV3.ip1) or day 16 (OV-4), plates were washed twice, cells were lysed (Reporter Lysis Buffer, Promega, Madison, WI) and freeze-thawed once. Protein concentration was measured with the DC Protein Assay system (Bio-Rad Laboratories, Hercules, CA).

Cervical cancer cell lines Caski, Hela, SiHa and C33A cells were infected as above. Cells were incubated in medium with 5% FBS, which was changed every other day. On day 13 (SiHa and C33A) or day 14 (Caski and Hela), MTS assay (CellTiter96®Aqueous One Solution Reagent, Promega, Madison, WI) was performed.
For the triple targeted oncolytic adenoviruses, cells were infected as above and incubated in medium with 5% FBS, half of which was changed every other day. On day 7 (nHEPs), day 9 (A549, BT474), day 10 (FHS173WE), day 14 (SKOV3.ip1), day 17 (OV-4) or day 18 (Hey and ES-2), MTS assay (CellTiter96®Aqueous One Solution Reagent, Promega, Madison, WI) was performed.

3.6 Quantitative PCR (II, III, IV)

Primary ovarian adenocarcinoma cells (II) were purified from malignant ascites samples as described (Barker, Casado et al. 2001). Analysis and creation of spheroids from primary ovarian carcinoma cells is detailed elsewhere (Lam, Kanerva et al. 2001). Briefly, purified unpassaged cells were incubated overnight in a 3% agar coated flask on a rocker to form spheroids, *ie.* three-dimensional clusters of cells. The spheroids were resuspended and infected with 1000 VP/cell of Ad5-Δ24RGD. Next day, spheroids were divided into 5 equal aliquots of 1 x 10^5 cells, which were collected daily. DNA was extracted with QIAamp DNA Mini Kit (Qiagen Inc., Valencia, CA), and quantitative PCR for the E1 gene was performed with Lightcycler® methodology as described (Hemminki, Belousova et al. 2001). In order to display the negative control, readings below the assay's detection limit were set as 1 and other results are displayed relative to this control.
10^5 cells/well (III, IV) were cultured on 96-well plates and infected with 0, 1 and 10 VP/cell. Quadruplet wells were collected after 2, 4, 6, 8 and 10 days and snap-frozen. DNA was extracted with DNeasy Tissue Kit (Qiagen Inc., Valencia, CA) and quantitative PCR for the E4 gene was performed as described.(Hemminki, Belousova et al. 2001) E4 copy numbers were normalized to human β-actin.

Table 6. Primers and probes for quantitative PCR

Adenoviral E1		**Used in**
Forward	5'-AACCAGTTGCCGTGAGAGTTG-3'	II
Reverse	5'-CTCGTTAAGCAAGTCCTCGATACA-3'	II
Probe	5'-CACAGCCTGGCGACGCCCA-3'	II
Adenoviral E4		
Forward	5'-GGAGTGCGCCGAGACAAC-3'	III, IV
Reverse	5'-ACTACGTCCGGCGTTCCAT-3'	III, IV
Probe	5'-TGGCATGACACTACGACCAACACGATCT-3'	III, IV
Human ß-actin		
Forward	5'-TAAGTAGGCGCACAGTAGGTCTGA-3'	II, III, IV
Reverse	5'-AAAGTGCAAAGAACACGGCTAAGT-3'	II, III, IV
Probe	5'-CAGACTCCCCATCCCAAGACCCCA-3'	II, III, IV

3.7 Oncolysis assay for peripheral blood mononuclear cells (PBMC) (III)

On day 1, PBMC and C33A cells were plated at 150,000 cells per well on 96-well-plates with 100 µl medium containing virus at 0, 10, 100 or 1000 VP/cell. Cells were cultured at 37°C and 5% CO_2. MTS assay was performed on days 4 and 6. Results are displayed in proportion to uninfected cells.

3.8 Detection of viral replication in PBMC (III)

10^5 PBMC/well were cultured with 5 µg/ml Phytohemagglutinin-P (PHA-P, Sigma), 100 IU/ml recombinant human interleukin 2 (IL-2, Proleukin, Chiron, Emeryville, CA) or no stimulation, and infected with 1000 VP/cell of Ad5-Δ24RGD, Ad5-Δ24E3 or Ad5lucRGD, at 37°C with 7% CO_2. Quadruplet wells were collected 1 h, 4 and 6 days later and frozen. DNA was extracted with DNeasy Tissue Kit (Qiagen Inc., Valencia, CA) and quantitative PCR for the E4 gene was performed as described.(Hemminki, Belousova et al. 2001) E4 copy numbers were normalized to human β-actin.

3.9 Tritiated thymidine uptake in PBMC (III)

PBMC were plated in quadruplicate with 10^5 cells/well and stimulated with 5 µg/ml PHA-P or 100 IU/ml IL-2. The mixture was infected with 1000 VP/cell of Ad5-Δ24RGD, Ad5-Δ24E3 or Ad5lucRGD in a total volume of 200 µl. Cells were cultured at 37°C with 7% CO_2. On days 3 and 5, cells were pulsed with 1 µCi of tritiated thymidine (New England Nuclear, Boston, MA) 18 - 20 h prior to harvesting with a Skatron Micro96 Harvester, and cell-incorporated tritium was assessed using a Packard Matrix 9600 Beta Counter. Results were expressed in counts per minute and converted to a stimulation index relative to the wells with no stimulation and no virus.

3.10 Detection of viral replication with TCID50 (IV)

$1.5x10^4$ cells/well of SKOV3.ip1 or BT474 were cultured on 96-well plates and after 24 hours infected with 10 VP/cell. Quadruplet wells (cells and supernatant) were collected after 2, 4, 6 and 8 days and carried over to 48-well plates. Cells were lysed with three freeze-thaw cycles. TCID50 was performed as published with 10^4 cells/well of 293 cells with dilutions up to 10^{-13} and detected after 10 days REF.

4. Preclinical in vivo evaluation of viruses

4.1 Therapeutic ovarian carcinoma model (II)

CB17 SCID mice (n=11/group) were injected with 1 x 10^7 SKOV3.ip1 cells ip. on day 0. On days 4, 5 and 6, mice were injected ip. with 1 x 10^{10} or 5 x 10^8 VP of Ad5-Δ24RGD, Ad5lucRGD (non-replicative control) or no virus. The virus was diluted with Opti-MEM into 1 ml in each case. Mice were inspected daily and euthanasia was performed in case of discomfort or distress. Survival data was plotted into a Kaplan-Meier curve and, using the LIFETEST procedure in SAS v.8.2 VENDOR, and the Ad5-Δ24RGD group was compared to the other groups with the log-rank test. The distribution of the data best fit the Weibull model, which was utilized for individual comparisons between the Ad5-Δ24RGD groups and controls, using the chi-square test of SAS v.8.2 LIFEREG procedure.

4.2 Cervical cancer animal model (III)

Female CRL.Nu/Nu mice (Charles River Laboratories, Wilmington, MA) (n=5/group) were injected in both flanks with $1x10^7$ C33A cells subcutaneously and tumors were allowed to grow. After 21 days viruses were injected i.t. or i.v., and tumor size was measured during follow up. The viruses were diluted with Opti-MEM into 200µl in each case. Mice were inspected daily and euthanasia of the complete group was performed in case of high tumor load, evident pain or distress. Studies were approved by the UAB Institutional Review Board.

4.3 Biodistribution of intravenous Ad5-Δ24RGD (III)

C33A tumors were inoculated in nude mice as above (n=3/group). At day 21, $5x10^{10}$ VP of Ad5-Δ24RGD diluted in 200µl OptiMEM was injected i.v. into each mouse. On days 1 and 7 after injection, liver, spleen, kidneys, heart, lungs and tumors were collected and snap-frozen with dry-ice/ethanol. DNA was extracted and *E4* copy number was determined as above.

4.4 In vivo imaging and survival analyses (IV)

Hey ovarian cancer cells were grown s.c. in nude mice and when tumors were ca. 10 mm^3, $3x10^8$ VP of each CRAD was injected on 3 consecutive days. Tumor size was followed and plotted relative to initial size. $3x10^8$ VP of luciferase expressing, non-replicating Ad5/3Luc1 was coinfected with each virus and *in vivo* luciferase imaging was performed 3 days later. For imaging, 4.5 mg of d-luciferin (Promega) was injected i.p in 100ul of 0% growth medium and images were captured with the IVIS 100 system using Living Image v. 2.50 (Xenogen inc., Alameda, CA).

SKOV3.ip1 ovarian cancer cells were injected with 10^7 cells per mouse and tumors were allowed to grow intraperitoneally for 7 days. Then 10^8 VP of Ad300wt, Ad5/3d24 or Ad5/3Cox2Ld24 were injected on three consecutive days intraperitoneally and survival was monitored.

5. Statistics (II, III, IV)

The ANOVA F-test (II) (SAS v.8.2, SAS Institute, Cary, NC) was performed to see if there were differences between the oncolytic potency of the viruses. If there was unequal distribution of the results, a two-sided t-test was utilized to assess statistical significance in comparison to the non-replicating virus.

An analysis of the tumor size data (III) was performed using a repeated measures growth model with PROC MIXED (SAS ®Ver. 8.02). Tumor size data was log transformed for normality. The effects of treatment group, time and the interaction of treatment group and time were evaluated by F tests. Baseline tumor size was included as a covariate in all models. *A priori* planned comparisons of adjusted differences in predicted treatment means were computed by t-statistics averaged over all timepoints. For all analyses a two-sided p value of < 0.05 was deemed statistically significant.

The F-test (IV) was performed to see if there were differences between the oncolytic potency of the viruses. If there was unequal distribution of the results, a two-sided t-test was utilized to assess statistical significance in comparison to the non-replicating virus, the Delta24 5/3-chimeric virus without TSP and the wt-virus controls. For all analyses a two-sided p value of < 0.05 was deemed statistically significant. *In vivo*, mean tumor size and standard deviations were calculated for each group of animals for each time point. A non-parametric change-point test was used to show a systematic change in the pattern of observations as opposed to fluctuation due to chance. The Proc Mixed procedure in SAS v.6.12 (SAS Institute, Cary, NC) was used to examine the effects of group and time on tumor growth. Pairwise comparisons were performed to compare groups.

RESULTS AND DISCUSSION

1. Transcriptional targeting of teratocarcinoma cells with the flt-1 promoter (I)

Flt-1, a receptor for vascular endothelial growth factor (VEGF), has been reported to display dysregulated expression in both tumor vasculature and tumor cells *per se* (von Marschall, Cramer et al. 2000; Bellamy, Richter et al. 2001; von Marschall, Cramer et al. 2001), suggesting that the flt-1 promoter might be a useful candidate to achieve tumor-specific transgene expression.

We demonstrated endogenous flt-1 expression in teratocarcinoma cell lines but not in ovarian, breast and cervical carcinoma cell lines. These data suggest that the expression of flt-1 mRNA reported in ovarian adenocarcinoma tumor blocks (Abu-Jawdeh, Faix et al. 1996) was likely caused by non-teratocarcinoma cells, such as endothelial cells of the tumor vessels. In contrast to our findings are the results of Masood et al. (Masood, Cai et al. 2001), who found a variety of cancer cell lines positive for flt-1 expression with RT-PCR, including the ovarian adenocarcinoma cell line Hey. A possible reason for the discrepancy could relate to the cells (eg. passage number) or be associated with the sensitivity of the RT-PCR assays used.

Although teratocarcinomas are rare tumors and usually treatable with chemotherapy (Gershenson 1993; Lu and Gershenson 2005), these findings are of potential interest since they represent the first time a promoter has been demonstrated to be active in teratocarcinoma, independent of the tissue type it originates from. We saw flt-1 promoter activity irrespective if the teratocarcinoma cell was derived from ovarian or testicular tissue. Any promoters that retain fidelity when placed in the Ad genome, achieve good levels of transgene expression and have a "liver off" phenotype are potentially of clinical utility. The close correlation of transgene expression and flt-1 mRNA shown here is further evidence that this promoter has the required characteristics. These data suggest that flt-1 could be useful for transcriptionally targeting teratocarcinoma.

The biological reason for the flt-1 promoter activity, we found in teratocarcinoma cells, remains unknown. Since teratocarcinomas are derived from undifferentiated, pluripotent early embryonal cells and contain cells from endo-, meso- and ectoderm (Damjanov 1993), lack of differentiation could be a possible reason for the expression of this promoter normally active in mature endothelium. In fact, previous studies suggested cross dependance of flt-1 gene expression and differentiation status (Bednarz, Weich et al. 1995; Bednarz, Weich et al. 1996). To test this hypothesis, we induced artificial differentiation of the teratocarcinoma cell lines with retinoid acid and cAMP or valproic acid (Imperiale, Kao et al. 1984; Werling, Siehler et al. 2001), followed by infection with a luciferase expressing adenovirus. Although we could validate artificial differentiation by observing changes in CMV driven gene expression and microscopic appearance of the cells, neither experiment demonstrated an association between flt-1 expression and artificially induced differentiation. However, both models used here are distinct from natural differentiation processes and thus may offer limited insight in this context.

2. Killing of ovarian cancer cells with conditionally replicating adenovirus Ad5-Δ24RGD (II)

In all cell lines, the crystal violet based cell killing assay showed replication of Ad5-Δ24RGD and Ad5wtRGD, followed by oncolytic death of cells. In the adenocarcinoma lines (Hey, SKOV3.ip1 and Ov-4), the CRAD replicated to similar degree as a virus containing wild type

early genes (Ad5wtRGD). For PA-1, a teratocarcinoma line, the oncolytic effect of Ad5wtRGD was slightly stronger than Ad5-Δ24RGD. Ad5LucRGD did not cause oncolysis. The crystal violet findings were confirmed with a quantitative assay based on mitochondrial activity, which reflects the amount of cells left after replication and oncolysis. When 10 VP/cell of Ad5-Δ24RGD was used for infection of Hey, SKOV3.ip1, PA-1 and OV-4 cells, 11.2%, 46.2%, 73.0% and 46.7% of cells remained alive (as compared to uninfected wells). For Ad5wtRGD, the positive control, the results were similar (13.7%, 22.7%, 28.0%, 51.9%). Therefore, Ad5-Δ24RGD has similar oncolytic potential to an E1 wild type virus.
Ovarian cancer primary cells spheroids provide a useful three-dimensional model for assessing replicativity of CRADs. More importantly, they provide a convenient means of maintaining primary cells alive in culture, without the confounding effect caused by clonal selection pressure involved in passaging and adaptation to cell culture. Spheroids were collected 1 thru 5 days after infection and quantitative PCR was performed to detect viral copies. One day after infection, 1.13 copies/well were detected, and the number grew exponentially to 1,036 – 19,336 – 402,000 – 4,296,000 copies on days 2 - 3 - 4 - 5. Thus, Ad5-Δ24RGD infects and replicates in primary unpassaged ovarian cancer cells. These results suggest that Ad5-Δ24RGD can infect and replicate in ovarian cancer cell lines and primary ovarian cancer cells.

3. Therapeutic efficacy of conditionally replicating adenovirus Ad5-Δ24RGD in a murine model of ovarian cancer (II)

We utilized an orthotopic murine model of ip. ovarian carcinomatosis and treated the mice with 3 ip. doses of 1 x 10^{10} VP of Ad5-D24RGD, the non-replicative Ad5lucRGD or no virus. Median survival was 64, 45 and 36 days, respectively and mean survival times for Ad5lucRGD and no virus were 45.7 and 37.6 days, respectively. Statistical analysis with the log-rank chi-square test indicated that survival was significantly better in animals treated with Ad5-Δ24RGD ($P<0.0001$). A smaller dose of the viruses (5 x 10^8 VP/day) was also investigated. The median survival was not reached for Ad5-Δ24RGD. For Ad5LucRGD and no virus, the median survival was 40 and 36 days and means were 41.9 and 37.6 days, respectively. All mice in the control groups expired before day 60. All mice treated with 5 x 10^8 VP of Ad5-Δ24RGD survived until at least day 61. The log-rank and chi-square tests confirmed that survival was significantly better in animals treated with Ad5-Δ24RGD ($P<0.0001$). Interestingly, none of the mice treated with Ad5-Δ24RGD showed evidence of i.p. disease after treatment. Instead, many developed subcutaneous tumors at the site where tumor cell injection had been performed, which eventually necessitated sacrificing of the animals. This could have been caused by a small number of tumor cells contaminating the needle tract during injection of cells. The virus probably had little access to the subcutaneous tissue and therefore could not eradicate these cells. It should be noted that no cures or long term survival had previously been reported for this aggressive model of ovarian cancer. All animals in the control groups expired or were killed due to i.p. tumor growth.
With the smaller dose of 5 x 10^8 VP daily for three days used here, ca. 1 x 10^{12} VP for a 60 kg human (w/w) were equaled. dl1520 has been administered to humans i.p. with doses ranging from a daily dose of 1 x 10^{11} to 1 x 10^{13} VP for 5 consecutive days (Kirn 2001). The results of this trial suggest that patients with bulky tumors (>2 cm) experienced dose limiting side effects at 1 x 10^{12} VP x 5, while patients with non-bulky tumors tolerated 1 x 10^{13} VP x 5 without toxicity. While comparisons between mouse and human data should be avoided, since

human Ads do not replicate in murine tissues to any significant degree, these figures suggest that the oncolytic potency of Ad5-Δ24RGD is sufficient to merit a human trial.

4. Killing of cervical cancer cells with conditionally replicating adenovirus Ad5-Δ24RGD (III)

In all four cervical cancer cell lines tested, the mitochondrial activity based MTS cell killing assay showed oncolysis mediated by Ad5-Δ24RGD and Ad300wt. When 10 VP/cell of Ad5-Δ24RGD was used for infection of C33A, Caski, Hela and SiHa cells, 0%, 24%, 16.5% and 34% of cells remained alive as compared to the uninfected control, whereas the wild type virus resulted in 0%, 6%, 11% and 29% living cells. Therefore, on cervical cancer cells *in vitro,* Ad5-Δ24RGD shows similar efficacy to wild type Ad.

Ad5-Δ24RGD was also compared to Ad5-Δ24E3, which is otherwise identical, but has a serotype 5 capsid. Similar or improved oncolytic potency was seen on all cell lines studied. This is in accord with findings of other investigators and our previous studies, demonstrating similar replicativity of CR2 deleted and wild type adenoviruses in tumor cells (Heise, Hermiston et al. 2000; Bauerschmitz, Lam et al. 2002).

5. PBMC can be infected but not lysed by conditionally replicating adenovirus Ad5-Δ24RGD (III)

Of special interest for possible human applications of Ad5-Δ24RGD is the effect on human leukocytes. Ad serotype 5 based agents do not effectively infect PBMCs in vivo (Wickham, Lee et al. 1997; Goossens, Havenga et al. 2001), although transduction can be forced with high titers in vitro (Schranz, Kulcsar et al. 1979; Chen, Ahonen et al. 2002). However, the RGD modification could increase the infectivity of PBMCs as these cells have been reported to express the relevant integrins (Horvath, Kulcsar et al. 1983). Ad capsids induce clonal increases in various PBMC lineages and there is migration of PBMCs to the infection site (Schranz, Kulcsar et al. 1979; Horvath, Kulcsar et al. 1983; Higginbotham, Seth et al. 2002). Thus, if PBMCs can be infected and sustain replication, there may be potential for hematological toxicity.

Freshly obtained PBMCs displayed no reduction in viability 4 or 6 days after infection with up to 1000 VP/cell of Ad5-Δ24RGD. This dose corresponds with a 10^{13} VP i.v. dose for a human adult with a blood count of $4.3x10^{9}$ leukocytes/liter, typical for cancer patients after chemotherapy (Grover 1996), and could therefore correlate with exposure in a clinical trial context. In contrast, the cervical cancer cells that were included as controls, were killed.

To mimic in vivo activation, PBMCs were infected in vitro with Ad5-Δ24RGD or controls and simultaneously stimulated with recombinant human IL-2 or PHA-P. Tritium incorporation was used as a marker of proliferation. On day 4, IL-2 and PHA-P had caused significant proliferation regardless of virus infection, although Ad5-Δ24RGD infected cells expanded slightly less. By day 6, these differences were increased indicating a possible toxic effect of Ad5-Δ24RGD. To determine if this was due to virus replication, virus copy number was determined in stimulated and unstimulated cells. No replication of any of the viruses was detected.

Importantly, we did not see any decrease in the viability of PBMCs following infection with Ad5-Δ24RGD. Further, no replication of the agent was seen in PBMCs regardless of stimulation. However, our results suggest that Ad5-Δ24RGD does interfere with IL-2 or PHA-P mediated stimulation. This was especially evident on day 6. The effect may be dependent on E1 expression in the cells, as the *E1*-deleted Ad5lucRGD did not produce

similar results. Further, as the isogenic control virus with a wild type adenovirus 5 capsid (Ad5-Δ24E3) did not cause this effect, it seems likely that RGD was required for entry. Despite probably entering PBMCs, Ad5-Δ24RGD was not able to replicate in or kill the cells, perhaps due to an intact Rb/p16 pathway or other cell type specific reasons.
This data is of special interest for possible human applications. It was shown, that Ad serotype 5 based agents do not effectively infect PBMCs *in vivo*,(Wickham, Lee et al. 1997; Goossens, Havenga et al. 2001) although transduction can be forced with high titers *in vitro*(Schranz, Kulcsar et al. 1979; Chen, Ahonen et al. 2002). However, the inserted RGD modification could have increased the infectivity of PBMCs as these cells have been reported to express the relevant integrins (Conron, Bondeson et al. 2001). Ad capsids induce clonal increases in various PBMC lineages and there is migration of PBMCs to the infection site(Schranz, Kulcsar et al. 1979; Horvath, Kulcsar et al. 1983; Higginbotham, Seth et al. 2002). Thus, if PBMCs could have been infected and subsequently sustained replication, there would be potential for hematological toxicity. Here 1000 VP/cell were used, an amount of virus corresponding to about 10^{13} VP i.v., similar to a CRAD dose tested in clinical trials (Bauerschmitz, Barker et al. 2002:Glasgow, 2004 #1833; Kanerva and Hemminki 2005).
Importantly, we did not see any decrease in the viability of PBMCs following infection with Ad5-Δ24RGD. Further, no replication of the agent was seen in PBMCs regardless of stimulation. However, our results suggest that Ad5-Δ24RGD does interfere with IL-2 or PHA-P mediated stimulation, which was especially evident on day 6. The effect may be dependent on E1 expression in the cells, as the *E1*-deleted Ad5lucRGD did not produce similar results. Further, as the isogenic control virus with a wild type adenovirus 5 capsid (Ad5-Δ24E3) did not cause this effect, it seems likely that RGD is required for entry. Despite probably entering PBMCs, Ad5-Δ24RGD was not able to replicate in or kill the cells, perhaps due to an intact Rb/p16 pathway or other cell type specific reasons.
Nevertheless, these experiments were designed as a preliminary investigation and further, more comprehensive studies are clearly required. Furthermore, while the information from preclinical experiments such as performed here, yield interesting and important data, clinical studies will ultimately determine the safety and efficacy of novel agents.

6. Biodistribution and *in vivo* replication of conditionally replicating adenovirus Ad5-Δ24RGD (III)

For performing a clinical trial, the best available preclinical assays should be used for evaluation of not only the efficacy, but also the safety of agents. Comprehensive murine toxicity studies with human serotype CRADs are not optimal, as human Ads do not productively replicate in mice (Higginbotham, Seth et al. 2002). Nevertheless, biodistribution can be assessed.
Eighteen hours after i.v. injection of Ad5-Δ24RGD into mice with s.c. tumors, virus was detected mainly in liver, spleen (35% compaired to the liver dose), lungs (25%) and tumor (14%), whereas heart (1.8%) and kidneys (1.4%) were infected with a lower amount. The liver to tumor ratio of virus copies was 7:1. On day 7 after injection, a 558-fold relative amplification of the virus within the tumor was found, suggesting i.t. viral replication *in vivo*. The dose of virus was chosen to allow direct comparison to previous experiments which were performed with *E1*-deleted Ads (Kanerva, Wang et al. 2002). Our results are well in accord with the data obtained with the RGD modified vector. As expected, the liver is the preferred organ for viral infection after i.v. injection. In contrast to i.p. injection, where spleen and liver have a similar viral uptake (Kanerva, Wang et al. 2002), only 35% of the liver dose reached

the spleen after i.v. injection. And whereas lung infection is insignificant after i.p. injection, 25% of the liver dose was found there after i.v. injection.
Human Ads do not productively replicate in most non-human cells and therefore it was an expected finding to see lack of replication of the agent in murine tissues (Higginbotham, Seth et al. 2002). In the liver, the virus DNA increased 3.5 fold (not significant), which may reflect a low degree of DNA multiplication without virion production.
More relevant toxicity studies could be performed if syngeneic model systems can be developed (Higginbotham, Seth et al. 2002). However, such systems would not be applicable to human viruses. Nevertheless, the respective non-human viruses could be constructed. Most recent publications reported replication of human adenoviruses to a certain degree in cottan rats, Syrian hamsters and swine (Toth, Spencer et al. 2005; Jogler, Hoffmann et al. 2006; Thomas, Spencer et al. 2006), but must be further investigated.

7. Therapeutic efficacy of conditionally replicating adenovirus Ad5-Δ24RGD in a murine model of disseminated cervical cancer (III)
The ultimate preclinical tests of experimental therapeutics are *in vivo* models. Here, we initially utilized as a proof of concept a murine model that mimics a strict locally recurrent advanced cervical cancer, a form of disease that would probably be accessible to local injection. Advanced C33A tumors were allowed to grow on the flancks. Then, mice were randomized into 6 groups and treated with three i.t. doses of either 10^7, 10^8, 10^9 or 10^{10} VP of Ad5-Δ24RGD, 10^{10} VP of the *E1*-deleted Ad5lucRGD or no virus in 200 µl OptiMEM. Treatment with the non-replicating Ad5lucRGD or without virus did not show an effect on tumor growth. Ad5-Δ24RGD at 10^{10} VP gave a significantly improved therapeutic effect over Ad5lucRGD and mock (both $P < 0.0001$). The same was true when the dose was 10^9 VP ($P < 0.0001$ *versus* Ad5lucRGD and $P = 0.0004$ *versus* mock) or 10^8 VP ($P = 0.0002$ *versus* Ad5lucRGD and $P = 0.0190$ *versus* mock). Interestingly, even the smallest dose injected gave a therapeutic effect in this aggressive model. The larger doses resulted in slightly higher efficacy. Converted weight/weight into a human dose, 10^7 VP would be approximately $2x10^{10}$, well below the $1x10^{13}$ VP of another CRAD that has been administered i.t. to humans without dose limiting side effects (DeWeese, van der Poel et al. 2001).
The most difficult form of cervical cancer to treat is disseminated disease. For such applications, systemic administration is necessary. Therefore, we evaluated i.v. injection of Ad5-Δ24RGD into mice bearing multiple tumors. Then, the mice were injected i.v. either with a single dose of 10^{10}, three doses of $3x10^{10}$ or a single dose of 10^{11} VP of Ad5-Δ24RGD. The controls were a single dose of 10^{11} VP of E1 deleted Ad5lucRGD and no virus.
I.v. injection of 10^{10} VP Ad5-Δ24RGD resulted in a slight reduction in tumor growth. At this dose, Ad5-Δ24RGD had an undramatic but statistically significant effect on tumor growth ($P = 0.0002$ *versus* Ad5lucRGD and $P = 0.0125$ *versus* mock). This is in accord with previous studies, where 1-2 x 10^{10} VP has been suggested as the threshold for uptake by the Kupffer cells of the liver (Alemany, Suzuki et al. 2000; Tao, Gao et al. 2001; Kanerva, Wang et al. 2002). In other words, only doses larger than this result in effective circulation of virus.
Fittingly, 10^{11} VP resulted in significant reduction of tumor growth, irrespective of whether a single or a split dose was used. The triple dose gave a more pronounced therapeutic effect over no virus, Ad5lucRGD or the lower single dose (all $P < 0.0001$). The same was true for the higher single dose (all $P < 0.0001$). There was no difference in efficacy between the triple and the higher single dose ($P = 0.3681$). Therefore, we were able to achieve therapeutic benefit utilizing systemic treatment in a murine model of disseminated cervical cancer.

There is limited data available on i.v. administration of CRADs to humans, but the trials that have been completed suggest good safety (Nemunaitis, Cunningham et al. 2001; Reid, Galanis et al. 2001; DeWeese, Arterbery et al. 2003; Hamid, Varterasian et al. 2003; Hecht, Bedford et al. 2003; Xia, Chang et al. 2004; Small, Carducci et al. 2006). The lack of demonstration of efficacy in early trials may be related to the low replicativity of the agents used (Barker and Berk 1987; Bischoff, Kirn et al. 1996; Hay, Shapiro et al. 1999), perhaps compounded by variable expression of CAR on target cells. In contrast, Ad5-Δ24RGD features replicativity similar to wild type, enhanced infectivity via metastasis associated integrins (Grill, Van Beusechem et al. 2001; Kawaguchi, Hosotani et al. 2001; Wesseling, Bosma et al. 2001; Hemminki, Zinn et al. 2002; Nakamura, Sato et al. 2002; Su, Liu et al. 2002) and independence from CAR. Considering the high prevalence of circulating neutralizing anti-adenovirus antibodies (Hemminki 2002), the capacity of RGD modified Ads to partially avoid pre-existing neutralizing antibodies may be useful (Blackwell, Li et al. 2000; Hemminki, Zinn et al. 2002). It is not known if the threshold effect associated with i.v. administration to mice applies to humans, or what the threshold dose might be. Obviously, this is of utmost importance for systemic application of CRADs and needs to be studied.
For performing a clinical trial, the best available preclinical assays should be used for evaluation of not only the efficacy, but also the safety of agents. Comprehensive murine toxicity studies with human serotype CRADs are not optimal, as human Ads do not productively replicate in mice (Hemminki, Kanerva et al. 2003). Nevertheless, biodistribution can be assessed. 18 h after i.v. injection, the liver to tumor copy number ratio was 7:1. 6 days later, a 558-fold i.t. copy number increase was seen. The dose of virus was chosen to allow direct comparison to previous experiments which were performed with *E1*-deleted Ads (Kanerva, Wang et al. 2002). Our results are well in accord with the data obtained with the RGD modified vector. As expected, the liver is the preferred organ for viral infection after i.v. injection. In contrast to i.p. injection, where spleen and liver have a similar viral uptake(Kanerva, Wang et al. 2002), only 35% of the liver dose reached the spleen after i.v. injection. And whereas lung infection is insignificant after i.p. injection, 25% of the liver dose was found there after i.v. injection. More relevant toxicity studies could be performed if syngeneic model systems can be developed (Hemminki, Kanerva et al. 2003). However, such systems would not be applicable to human viruses. Nevertheless, the respective non-human viruses could be constructed.
These data suggest, that Ad5-Δ24RGD can successfully infect tumor cells and replicate *in vivo*, even when the virus is injected i.v. Of note, none of the mice died or displayed visual evidence of toxicity after i.t. or i.v. viral injection.

8. Effect of combination of promoter and deletion on selectivity and efficacy of conditionally replicating adenoviruses (IV)

We constructed six novel viruses, each controlled by long or medium variants of the cox2 promoter (Yamamoto, Alemany et al. 2001; Yamamoto, Davydova et al. 2003). These promoter variants have been evaluated previously for potential utility for ovarian cancer gene therapy (Casado, Gomez-Navarro et al. 2001; Barker, Coolidge et al. 2003). The cox2 promoter has been suggested a potentially useful tumor specific promoter given low expression in livers of mice and relatively high expression in many tumor types including ovarian cancer (Casado, Gomez-Navarro et al. 2001; Barker, Coolidge et al. 2003; Yamamoto, Davydova et al. 2003; Kanerva, Bauerschmitz et al. 2004). Three variants of the central adenoviral replication regulator gene (E1A) were used: wild-type E1, a Delta24-modified version (24 bp deletion in Rb binding site of E1A) or a Delta2-Delta24-modified

version (additional incorporation of a 3 bp deletion – encoding for second amino acid of E1A, ie. the p300 binding site (Nettelbeck, Rivera et al. 2002). The specificity and efficacy of Delta24-modified viruses have been demonstrated previously (Fueyo, Gomez-Manzano et al. 2000; Bauerschmitz, Lam et al. 2002; Nettelbeck, Rivera et al. 2002; Kanerva, Zinn et al. 2003; Lam, Bauerschmitz et al. 2003; Lam, Kanerva et al. 2004). Theoretically, additional control over E1A might result in higher specificity allowing an improved therapeutic window (difference in replication between normal and cancer cells). An earlier publication suggested tumor specificity for the Delta2 mutation in the context of cervical cancer cells (Balague, Noya et al. 2001). All viruses constructed here were infectivity enhanced with the adenovirus type 3 fiber knob in the adenovirus type 5 fiber shaft. Our previous work suggests that this may be the "best currently available" transductional targeting moiety for ovarian cancer (Kanerva, Mikheeva et al. 2002; Kanerva, Wang et al. 2002; Kanerva, Zinn et al. 2003; Lam, Kanerva et al. 2004).

We compared two variants of the cox2 promoter; a longer version (cox2L, 1554 bp) which has been reported more specific, and a medium version (cox2M, 988 bp), which might feature a higher expression level (Yamamoto, Alemany et al. 2001; Yamamoto, Davydova et al. 2003). Here, we saw similar results in that the cox2L promoter was slightly more specific, especially in the context of wild type E1A. In tumor cells we did not see significant differences between the promoters, but there seemed to be a tendency for the cox2M promoter to allow slightly higher rates of cell killing when driving wild type E1 or driving Delta2-Delta24.

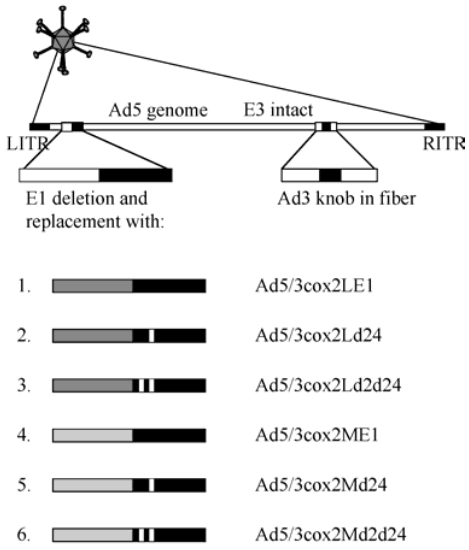

Fig. 6. Schematic illustration of the six different triple-targeted selectively oncolytic adenoviruses

When the different combinations of promoter with E1 variants were compared, Delta24 resulted in the most promising data. The rates of oncolysis were higher (cox2L) or similar (cox2M) to wild type E1A. Other studies have corroborated the high replicativity of Delta24

type viruses (Heise, Hermiston et al. 2000; Bauerschmitz, Lam et al. 2002). Oncolysis caused by Delta2-Delta24 was approximately 10-fold less than with the other versions of E1. This is in concordance to other publications showing that viruses with multiple modifications feature slower rates of replication (Alemany, Balague et al. 2000; Balague, Noya et al. 2001). Interestingly, we saw little or no additional specificity when the Delta2 mutation was added to Delta24. Thus, the double modified E1A does not seem to provide specificity benefits over the single modified E1A (Delta24), but does reduce efficacy.

The specificity of heterologous promoters can be affected by viral elements such as the inverted terminal repeats. Therefore, it is important to note that the specificity of the cox2 promoter seemed retained: in the low cox2 expressing cell line BT474 the rates of replication were 100-fold lower than with wild type. In contrast, in the cox2 positive cell line A549, replication of the CRAds was similar to Ad5 wild type, except for the double modified Delta2-Delta24, which was slower. A549 cells express CAR to high degree (Wu, Fernandez et al. 2001) and therefore this cell line allows comparison of the CRAds to Ad5wt in a situation where all viruses infect cells effectively.

Ovarian cancer cells have been reported refractory to infection with adenovirus. Therefore, we sought to transductionally target the novel CRAds by utilization of the serotype 3 knob. In OV-4 and SKOV3.ip1 cells Ad5wt resulted in little oncolysis, while the Ad5/3 CRAds were up to 1000-fold more effective in infecting and killing cells, due to higher expression of the serotype 3 receptor (Kanerva, Mikheeva et al. 2002). In Hey and ES-2 there was a ca. 10-fold difference in cell killing. The same was true for the fibroblast cell line FHS173WE, which displays little CAR but seems to express the adenovirus 3 receptor, because it allows viruses with the serotype 3 knob to enter cells. With incubation times similar to the cancer cell lines, no oncolysis was evident. However, when longer incubation times were used, killing was seen. To investigate this further, we are in the process of determining the cox2 expression of this fibroblast line, and if adenovirus infection can induce cox2 in these cells. Although it is difficult to interpret this with regard to potential toxicity, as most fibroblasts *in vivo* would not be dividing (as opposed to fibroblasts *in vitro*), we felt that this might be a stringent model for detecting potential differences with regard to specificity between the CRAds.

Interestingly, all 5/3 CRAds seemed to replicate in FHS173WE cells, and the promoter increased the selectivity of the virus significantly compared to Ad5/3d24 ($p<0.01$ at 1 VP/cell) with a 10-fold difference in cell killing.

In addition, we demonstrated that the cox2 promoter driven CRAds killed tumor cells with comparative or even superior efficacy when compared to Ad5/3d24, which is the isogenic Delta24 virus with the serotype 3 knob. This is most likely due to a higher amount of E1A proteins produced, when expressed from the cox2 promoter. This could improve replication, or increase cell killing might be due to the anti-tumor effect of E1A proteins *per se* (Hortobagyi, Ueno et al. 2001). In non-replicating fibroblasts replication of the cox2 driven CRAds was reduced and none of the tested viruses lysed human peripheral blood mononuclear cells (PBMC) which might be useful from a safety standpoint (Bauerschmitz, Kanerva et al. 2004).

As the liver is an important organ with regard to toxicity from adenoviral gene therapy (Raper, Chirmule et al. 2003), we evaluated primary human hepatocytes (nHEPs) with regard to replication permissivity. None of our CRAds replicated in these cells, resulting in at least 100-fold difference in replication compared to Ad5 wild type. These results suggest the potential for low liver toxicity. Nevertheless, in addition to hepatocytes, human livers contain other cell types such as endothelial cells and specialized macrophages known as Kupffer cells. Also, as the tertiary structure of the liver may also be important with regard to adenoviral gene transfer, more advanced models or clinical trials are needed to reliably evaluate toxicity.

In conclusion, our results suggest increased specificity and efficacy for viruses featuring the cox2 promoter with the Delta24 deletion, in comparison to viruses without the deletion. Further, CRAds with Delta24 seemed advantageous over viruses with Delta2-Delta24 due to stronger replication, while the specificity was similar. Further, cox2Delta24 was more oncolytic than cox2E1 or Ad5/3d24. When data from ovarian cancer cells (cox2-Delta24 100-fold more effective) are combined to data from hepatocytes (Ad5wt 100 - 1000 fold more toxic), the cox2Delta24 configuration increased the therapeutic window by 10 000 – 100 000 fold in comparison to Ad5wt. With regard to promoter configuration cox2L was more specific and effective than cox2M. Therefore, cox2L-Delta24 emerges as the agent of choice for further studies which may eventually facilitate clinical testing of the agent.

9. Therapeutic efficacy of triple-targeted selectively oncolytic adenoviruses in vivo (IV)

The ultimate preclinical tests of experimental therapeutics are *in vivo* models. Ovarian cancer cell xenografts were established subcutaneously and injected with viruses. Ad5/3Cox2Ld24 had more antitumor effect than the positive control Ad300wt (P=0.0001), and was more effective than negative controls Ad5LacZ (P<0.01) and mock (P<0.001), and had similar efficacy to Ad5/3d24 (P=0.4). In this aggressive model, Ad5/3Cox2Ld2d24 and Ad5/3Cox2LE1 were not more effective than negative controls (P not significant).

To evaluate replication of the viruses *in vivo*, CRADs were coinfected with a luciferase expressing virus (Ad5/3Luc1), which replicates only in the presence of E1 produced by replication of the CRADs. In comparison to tumors coinfected with replication deficient virus (Ad5LacZ), tumors coinfected with CRADs demonstrated more luciferase expression (all P<0.01).

Finally, to mimic advanced stage ovarian cancer in an orthotopic animal model, SKOV3.ip1 ovarian cancers were established intraperitoneally with 10^7 cells/mouse. Tumors were allowed to grow for 7 days and then mice were injected with 10^8 VP/day or mock for three consecutive days. After 90 days Ad300wt (P=0.002), Ad5/3Cox2Ld24 (P<0.001) and Ad5/3d24 (P<0.001) resulted in significant longer survival than mock. Ad5/3Cox2Ld24 (P=0.120) and Ad5/3d24 (P=0.148) also displayed a trend for more antitumor effect than the positive control Ad300wt, but the differences were not statistically significant Ad5/3Cox2Ld24 had similar efficacy to Ad5/3d24 (P=0.971). Again, cox2L-Delta24 emerges as the agent of choice for further studies which may eventually facilitate clinical testing of the agent.

SUMMARY AND CONCLUSIONS

In this thesis, the evolution of adenoviral therapy is described from its early beginnings with poorly characterized preparations of wild type viruses to non-replicative adenoviral vectors to most recent generation of multiple modified conditionally replicating adenoviruses.

Any promoters that retain fidelity when placed in the Ad genome, achieve good levels of transgene expression and have a "liver off" phenotype are potentially interesting for clinical testing. We showed activity of the flt-1 promoter in teratocarcinoma cells derived from ovarian or testicular tissue. The close correlation of transgene expression and flt-1 mRNA seen is further evidence that this promoter may have the required characteristics. The data, obtained in the current study, suggest that flt-1 could be useful for transcriptionally targeting teratocarcinoma.

Further, we have then utilized a replication competent agent, Ad5-Δ24RGD, in ovarian cancer models. We observed replication and oncolytic potency similar to a wild type control virus for ovarian cancer cell lines and in a three dimensional spheroid model. We detected an exponential increase in the amount of Ad5-Δ24RGD gene copies suggesting efficient replication. Finally, in an orthotopic murine model of peritoneally metastatic ovarian cancer we saw significant improvement in survival of the animals with complete eradication of ip. disease. Thus, Ad5-Δ24RGD could be an effective agent for treatment of ovarian cancer, and because Rb/p16 pathway defects are ubiquitous in human cancers, we sought to extend these studies to cervical cancer. Ad5-Δ24RGD was administered locally and systemically. Therapeutic efficacy was seen with amounts of virus (converted into human doses) that seem amenable to clinical testing, and have been safe in trials with other CRADs. These results suggest that Ad5-Δ24RGD may be a useful agent for testing in clinical trials with ovarian or cervical cancer patients suffering from disease refractory to current treatment modalities.

Although the safety of Ads has been good in human trial heretofore, the emergence of more effective, tropism modified and replication competent agents suggests that improving specificity might be useful. We utilized two variants of the cox2 promoter in combination with E1-transcomplementation and found increased specificity and efficacy for viruses featuring the cox2 promoter with the Delta24 deletion, in comparison to viruses without the deletion. Further, CRAds with Delta24 seemed advantageous over viruses with Delta2-Delta24 due to stronger replication, while the specificity was similar. Impressively, cox2Delta24 was similar or even more oncolytic than cox2E1 or Ad5/3d24. When data from ovarian cancer cells (cox2-Delta24 100-fold more effective) are combined to data from hepatocytes (Ad5wt 100 - 1000 fold more toxic), the cox2Delta24 configuration increased the therapeutic window by 10 000 – 100 000 fold in comparison to Ad5wt. With regard to promoter configuration cox2L was more specific and effective than cox2M. Therefore, cox2L-Delta24 emerges as the agent of choice for further studies which may eventually facilitate clinical testing of the agent.

In conclusion, effective but specific tumor transduction continue to be the limiting steps for adenoviral vectors, and advanced generation replicating agents like CRAds are emerging as promising agents for overcoming some of the obstacles. One central problem has been that the primary receptor, CAR, is often expressed at variable levels on primary tumor tissue whereas in normal tissue and especially the liver its levels are high. Thus, targeting strategies and infectivity enhancements methods have been developed and promising preclinical data sets the stage for clinical testing. Though other viral vectors might be more useful for treatment of hereditary and acquired diseases, adenoviruses are highly promising and safe agents for

oncology, as suggested in number of clinical trials. Importantly, synergism and a lack of cross resistance of CRAds with chemotherapy or radiation therapy has been demonstrated. Thus, combination treatments seem lucrative for testing in trials.

ACKNOWLEDGEMENTS

This work was carried out between 2000 and 2007, partially done at the Department of Obstetrics and Gynecology in Düsseldorf University Medical Center, at the Division of Human Gene Therapy of the University of Alabama at Birmingham, and last but not least finished in the Cancer Gene Therapy Group at the University of Helsinki.
I wish to express my sincere gratitude to the numerous people who participated in and contributed to this project in many different ways:

I wish to thank Professor Risto Renkonen, Coordinator of the Rational Drug Design Program, and Professor Pekka Häyry, Head of the Transplantation Laboratory, Academy Professor Kari Alitalo, Coordinator of the Molecular Cancer Biology Program, Professor Seppo Meri, Head of Haartman Institute, Professor Olli Jänne, Head of Biomedicum, and Dean Matti Tikkanen, for providing excellent research facilities in Helsinki.

Professor Jorma Paavonen is warmly thanked for accepting the important post of custos at the dissertation.

Professor H.G. Bender and Professor Peter Dall from the Department of Obstetrics and Gynecology at Düsseldorf University Medical Center for giving me the perfect start in clinical and scientific work and all their valuable support during the last years.

Professor David T. Curiel, Director of the Gene Therapy Center at UAB, for the opportunity to join his excellent group without knowing me or my non-existing scientific background. I wish to thank him for giving me not only excellent resources but also his enormous knowledge of Gene Therapy. Together with his wife Gloria, they arranged support to settle in the “un-European” world of Alabama, send me to meetings all over the US and let me get know their family and traditions. Both I owe great thanks.

My supervisor docent Akseli Hemminki, for teaching me scientific thinking, guiding me skillfully throughout this study, and always finding something good in my results. He patiently taught me English academic writing and scientific thinking. I wish to thank him for his continuous support in Birmingham, Düsseldorf and Helsinki.

As a second supervisor I was taught by Anna Kanerva the basic skills of adenoviral lab work, and I am especially thankful for all discussion and great advises I got from her through the years.

Docent Seija Grenman and Professor Arto Urtti, the official reviewers of this thesis, for critical review and most valuable comments on the manuscript.

The members of Cancer Gene Therapy Group (CGTG) at University of Helsinki: Akseli, Anna, Tanja, Minna, Sari, Aila, Annina, Eerika, Hanna, Iulia, Joao, Lotta, Mari, Maria, Merja, Kilian, Petri, Sergio,and Tuuli for providing a lab anyone would wish to work in. There are not many places where work makes so much fun. I will especially miss morning-coffee- and afternoon-tea-discussions.

All my colleagues from the Department of OB/GYN in Düsseldorf for giving me more than once the chance to take time off from the clinics for research. My Düsseldorf research colleagues PD Dr. Daniel Rein, Dr. Dieter Niederacher, Dr. Isabel Herrmann and Arthur Menzel for providing an atmosphere in which I was still willing to do research after long hours in the clinic.

My colleagues at the Division of Human Gene Therapy in Birmingham. Especially I wish to thank here Anna, Aksu, Daniel, Dirk, John, Shannon, Taco and Tanja. We were not only colleagues but also friends. You provided me the feeling of being at home in Alabama and made the two years most valuable for me.

My collaborators at UAB, University of Glasgow, Institut Catalana d'Oncologica in Barcelona ,University of Kuopio and University of Erlangen for sharing their knowledge and reagents.

My friends in Finland, Anna, Aksu and Tanja, who provided me together with their families all support needed to come to Helsinki. Aksu especially I have to thank for developing the idea of obtaining a PhD in Finland at five in the morning at Tanja's PhD-party and helping me to find funding for this plan. I am happy to have had Tanja as "my princess" in the lab, and Anna as my soulmate in how things should be done correctly. Probably together we were hard to stand for other people every now and then.

All my friends in Germany. In particular Stephan, Robert, Kerstin, Florian, Ralf, Gisela, Klaus, Daniel, Martina and Nina, for being supportive and "just there" every time I needed them, although I liked to hide myself behind work every time I did not want to meet anybody.

My family; my late father Heinz for believing in me and making me independent from financial limitations with regard to my career. My mother Marianne for being the best mom in the world, supporting me in all my weird ideas without questioning and being the most strict reviewer ever. My younger brother Klaus for being always "back-up" and "on call" when I wished to overcome time limitations as well as taking care of my apartment when I spent time abroad, and my older brother Carl for all the inspiration he gave me.

I am very grateful for the financial support provided by: Deutsche Forschungsgemeinschaft (DFG), EU framework project 6, the Sigrid Juselius Foundation, the Academy of Finland, Helsinki University Central Hospital Research Funds, University of Helsinki Internal Funds, the Sohlberg Foundation, Biocentrum Helsinki, the Finnish Cancer Society, the Emil Aaltonen Foundation, the Maud Kuistila Foundation, the Finnish Medical Foundation, the Ida Montin Foundation, the Instrumentarium Research Fund, the Biomedicum Helsinki Foundation, the Finnish Oncology Association, the Research and Science Foundation of Farmos, United States Army Department of Defense Contract, The Lustgarten Foundation and the NIH

Helsinki, in spring 2007

Gerd Johannes Bauerschmitz

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